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Docket No.: 28053/38258
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Patricia L. Conway et al.

Application No.: 09/889,085 Confirmation No.: 6842

Filed: January 9, 2002 Art Unit: 1651

For: IMPROVED MICROBIAL PREPARATIONS Examiner: V. Afremova

APPEAL BRIEF

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

As required under § 41.37(a), this brief is filed more than two months after the Notice of Appeal filed in this case on September 29, 2005, and is in furtherance of said Notice of Appeal.

The fees required under § 41.20(b)(2), and any required petition for extension of time for filing this brief and fees therefor, are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

This brief contains items under the following headings as required by 37 C.F.R. § 41.37 and M.P.E.P. § 1206:

I.	Real Party In Interest
II	Related Appeals and Interferences
III.	Status of Claims
IV.	Status of Amendments
V.	Summary of Claimed Subject Matter
VI.	Grounds of Rejection to be Reviewed on Appeal

I. REAL PARTY IN INTEREST

The real party in interest for this appeal is:

PENFORD HOLDINGS PTY LTD

II. RELATED APPEALS, INTERFERENCES, AND JUDICIAL PROCEEDINGS

There are no other appeals, interferences, or judicial proceedings which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

III. STATUS OF CLAIMS

A. Total Number of Claims in Application

There are 79 claims pending in the application.

B. Current Status of Claims

1. Claims canceled: 1-19, 76

2. Claims withdrawn from consideration but not canceled: 20-75

3. Claims pending: 41, 76-153

4. Claims allowed: none

5. Claims rejected: 41, 76-153

C. Claims On Appeal

The claims on appeal are claims 41, 76-153

IV. STATUS OF AMENDMENTS

Applicant filed an Amendment After Final Rejection on August 1, 2005. The Examiner responded to the Amendment After Final Rejection in an Advisory Action mailed September 8, 2005. In the Advisory Action, the Examiner indicated that Applicants' proposed amendments canceling claims 41 and 76 would not be entered.

Accordingly, the claims enclosed herein as Appendix A do not incorporate the amendments canceling claims 41 and 76 as indicated in the paper filed. However, the claims in Appendix A do incorporate the amendments indicated in the paper filed by Applicant on February 22, 2005.

Nevertheless, Appellants wish to withdraw from consideration on appeal claims 41 and 76 to simplify the issues presented on appeal.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Applicants' invention relates to the discovery that harvested microbes which have been previously cultured in or grown on resistant starch and then subsequently incorporated into a product have improved viability and survival/recovery rates (compared with the same microbes cultured in or grown on a medium not containing resistant starch). Thus, for example, *Bifidobacteria* previously grown on media which contained resistant starch has a superior survival/recovery rate compared to the same strain of *Bifidobacteria* previously grown on media which did not contain resistant starch.

Each of independent claims 77, 79, 81 and 88 from which all the remaining appealed claims depend recite "[a] microbial preparation comprising harvested microbes which have

been grown or cultured in a media based on or containing resistant starch (page 3, lines 13-16) in a manner such that when subsequently incorporated in a product, the survival/recovery rate of the harvested microbes is increased as compared with the same microbes grown or cultured in a media without resistant starch, (page 3, lines 9-11) the product being selected from the group consisting of a food, feed, nutraceutical, biocontrol and bioremediation product (page 3, lines 12-13)...[wherein the resistant starch is a resistant starch from one of several selected categories]." Those categories are:

for independent claim 77: "RS1, RS3 or RS4" (page 7, line 6);

for independent claim 79: "[starch derived from] rice, barley, wheat, legumes, bananas" (page 7, lines 9-13);

for independent claim 81: "derived from a starch having an amylose content of at least 40%"; (original claim 6, page 22, lines 15-18) and

for independent claim 88: "starch [which] is chemically, physically, and/or enzymically treated or modified." (original claim 11, page 22, lines 26-28)

VI. GROUNDS OF OBJECTION TO BE REVIEWED ON APPEAL

A. Whether claims 77, 79, 81, 88, 90-105, 109-120, 124-135 and 139-150 are unpatentable under 35 U.S.C. § 102(b) as being anticipated by Masuda, U.S. Patent 5,143,845.

B. Whether claims 77-153 are unpatentable under 35 U.S.C. § 102(b) as being anticipated by Brown et al., U.S. Patent 6,060,050 ("Brown '050") in light of evidence by McNaught et al., U.S. Patent 5,714,600.

C. Whether claims 77-153 are unpatentable under 35 U.S.C. § 103(a) as being unpatentable over Masuda taken with Brown et al. Food Australia 50(12) (Dec. 1998) ("Brown, Food Australia") and McNaught et al.

D. Whether claims 77-153 are unpatentable under the judicially created doctrine of obviousness-type double patenting over claims 1-12 of Brown et al., U.S. Patent 6,221,350 ("Brown '350").

VII. ARGUMENT

Applicants' invention relates to the discovery that harvested microbes which have been previously cultured in or grown on resistant starch and are then subsequently incorporated into a product have improved viability and survival/recovery rates compared with the same microbes cultured in or grown on a medium not containing resistant starch. Thus, for example, *Bifidobacteria* previously grown on media containing resistant starch has a superior survival/recovery rate compared to the same strain of *Bifidobacteria* previously grown on media which does not contain resistant starch.

It is hypothesized that these improvements in the harvested bacteria are due to some biochemical change in the microbes themselves. However, as no conventional structural limitation to the microbes themselves can be added to the claims, the microbes can only be defined by the process steps by which they are made. Thus, Appellants have defined the microbes using process limitations which define how the novel microbes are produced. These process steps impart distinctive structural characteristics to the final microbes that manifest themselves in an improved survival/recovery rate. These improvements to the microbes are clearly evidenced by the examples presented in the specification.

These improvements to the microbes described above are clearly evidenced by the examples presented in the specification and are not challenged by the Examiner. Rather the appealed rejections are prior art rejections based on the arguments that the improved harvested microbe preparations existed in the prior art and are therefore anticipated. Alternatively, the rejections are based on the argument that the prior art taught the steps of culturing microbes on resistant starch and later harvesting them to be used as part of improved harvested microbe preparations.

One of the rejections ((A) the Section 102 rejection over Masuda) is premised on the proposition that the harvested microbes of the invention are identical to prior art microbes which were not harvested from resistant starch. Another of the rejections ((B) the Section 102 rejection over Brown '050 in view of McNaught) is premised on the misapprehension that fecal microbes growing on resistant starch in the gut were harvested; and still others ((C) the Section 103 rejection over the combination of Masuda with Brown '050, Brown, Food Australia and McNaught; and (D) the obviousness-type double patenting rejection over

Brown '350) are premised on the related misapprehensions that harvested microbes that are later co-packaged with resistant starch to produce a probiotic composition constitute or suggest the invention.

A. THE 35 U.S.C. §102(B) ANTICIPATION REJECTION OVER MASUDA ET AL. U.S. 5,143,845 SHOULD BE REVERSED BECAUSE THE MASUDA MICROBES WERE NOT GROWN ON RESISTANT STARCH AND MICROBIAL SPORES DO NOT ANTICIPATE APPELLANTS' CLAIMS.

The rejection over Masuda based on the argument that bacteria having enhanced heat, dry and drug stability after spore formation inherently anticipate the claimed products should be reversed because the improvements in survival/recovery rate manifested by the claimed microbes are not the same as or the result of spore formation. Moreover, Masuda does not grow its microbes on resistant starch, and the symbiosis of three kinds of bacteria disclosed by Masuda is not the same as or relate to the improved survival/recovery of the claimed microbes.

Appellants have previously established by the submission of the Declaration of Ian Brown submitted February 25, 2005 (attached as Appendix B) that there was no resistant starch in the microbial culture media of Masuda and that the Appellants' claims are drawn to microbial preparations *per se*. Appellants acknowledge that product-by-process claims are limited only by the final structure of the product obtained but emphasize that the final structure of the microbial preparations claimed in their application is different from that of the spores disclosed by Masuda.

Product-by-process claims constitute a means by which Appellants can claim their inventions even where the nature of the invented product is such that it is difficult to define. Thus, the Supreme Court held in *Bonito Boats Inc. v. Thunder Craft Boats Inc.*, 489 U.S. 141, 9 USPQ 2d 1847, 1855 (1989) that "As long as the end product of the process is adequately defined as novel and nonobvious, a patent on the process may support a patent in the resulting product." Product-by-process claims are thus "perfectly acceptable [one] so long as the claims particularly point out and distinctly claim the product or genus of products for which protection is sought." *In re Brown and Saffer*, 173 USPQ 685, 688 (CCPA 1972). As stated in MPEP 2113, "the structure implied by the process steps should be considered when assessing the patentability of product-by-process claims over the prior art." The MPEP

continues by stating that this may be done by showing that the claimed product (microbe) exhibits unexpected properties compared with the prior art. This is supported by *In re Fessman*, 18 USPQ 324, 326 (CCPA 1974) in which the court states that it is "applicant's duty to present evidence which would demonstrate the unobvious character of his claimed invention over the cited reference."

Appellants have complied with the duties laid out for them by the courts by using the process steps to imply the structural difference of improved survival/recovery and then exemplifying the structural difference by comparing microbes cultured on resistant starch with those which were not.

Thus, the Application has provided evidence in the form of the various examples that microbes harvested from a resistant starch containing culture have improved survival and recovery compared to the same organisms harvested from media without resistant starch. (See each of Examples 1-11 and Figs. 1-14 corresponding thereto). Thus, it is clear that the products of the application are different from and represent an improvement over those of the prior art such as Masuda which are not grown in media containing resistant starch, and do not have this increased survival/recovery.

There is no evidence in Masuda which would indicate otherwise. Masuda is directed to symbiotic mixtures of lactic acid producing bacteria (LB), saccarifying bacteria (SB) and butyric acid producing bacteria (BB) as prebiotic components of probiotic compositions to be consumed. The Masuda compositions were not cultured on resistant starch but were only said to have enhanced heat, dry and drug stability after spore formation! (Col. 2, lines 53-55, col. 5, lines 2-4) Appellants' composition is not directed to microbial spores!

Moreover, the symbiosis reported by Masuda with respect to the combination of three different types of organisms (LB), (SB) and (BB) does not relate to improvements in survival/recovery rate of the microbes. Instead, Masuda describes its symbiosis of the microbes as being the "promot[ion] [of] their own growth in a living body by ... cooperative action" (col. 2, lines 6-7). Masuda proposes that a possible mechanism for the symbiosis as being "that in the course of cultivation respective bacteria will produce a growth promoting factor which is useful for each other." (col. 2, lines 27-30.)

Neither this symbiosis in growth nor the stabilization of spore formation anticipate Appellants' claims. Accordingly, the present invention is novel over Masuda and the rejections of claims 77, 79, 81, 90-105, 109-120, 124-135 and 139-150 over Masuda should be reversed.

B. THE REJECTION OF CLAIMS 77-153 UNDER 35 U.S.C. §102(B) OVER BROWN '050 IN VIEW OF MCNAUGHT ET AL. SHOULD BE REVERSED BECAUSE GROWING FECAL MICROBES ON RESISTANT STARCH IN THE GUT OR PLATING SUCH MICROBES DOES NOT ANTICIPATE APPELLANTS' CLAIMS.

The rejection of claims 77-153 under 35 U.S.C. § 102(b) as being anticipated by Brown, et al. (US 6,060,050) in the light of evidence by McNaught, et al. (US 5,714,600) should be reversed because Brown '050 is directed to probiotic compositions comprising combinations of microbes and resistant starch but is directed to the culturing of microbes on resistant starch in the gut! As such, Brown '050 neither harvests microbes grown on resistant starch nor puts microbes so grown into microbial preparations.

Brown '050 is directed to probiotic compositions for human consumption and evaluates the ability of fecal bacteria to grow in the gut in the presence of probiotic compositions containing resistant starch. Brown '050 discloses experiments modeling the behavior in the large bowel of such probiotic compositions comprising bacteria in the presence of various starch substrates and discloses their growth profiles but does not harvest the bacteria used in these experiments.

While the Examiner argues that Brown '050 "teaches microbial preparations grown on resistant starch," (citing Brown col. 5, lines 32-36 and figures 9 and 10) the reference only plates the microbial compositions in order to obtain a microbial population count. Brown '050 neither harvests the microbes nor puts them into products. Brown '050 grows fecal bacteria in order to count them!

As one skilled in the art knows, harvesting microbes typically involves separating them from the media. This not only concentrates the microbes, but also typically removes by-products of the proliferation (e.g. fermentation). In contrast, counting or enumerating microbes involves removing a small aliquot of microbes with its environment (in the case of Brown '050, with fecal material) and allowing it to proliferate to be counted. For example, by spreading on agar, each microbe develops a colony such that one can count the microbes

in the original fecal sample. Such plating does not anticipate Appellants' claims and the rejection of claims 77-153 under 35 U.S.C. § 102(b) as being anticipated by Brown '050 should be reversed.

C. THE REJECTION OF CLAIMS 41 AND 76-153 UNDER 35 U.S.C. §103(A) OVER MASUDA, BROWN '050, BROWN, FOOD AUSTRALIA AND MCNAUGHT SHOULD BE REVERSED BECAUSE MASUDA DOES NOT DISCLOSE CULTURING MICROBES ON RESISTANT STARCH AND THE PROBIOTICS OF BROWN '050 AND BROWN, FOOD AUSTRALIA DO NOT DISCLOSE OR SUGGEST THE HARVESTING OF MICROBES CULTURED ON RESISTANT STARCH

The rejection of claims 77-153 under 35 U.S.C. § 103(a) as being unpatentable over Masuda taken with Brown '050, Brown, Food Australia and McNaught should be withdrawn because no rationale has been provided why the disparate references should be combined.

As discussed above, Masuda is directed to spore formation and symbiotic microbe mixtures but does not teach the culturing of microbes on resistant starch. Brown '050, and Brown, Food Australia disclose probiotic combinations of microbes and resistant starch but such probiotics do not constitute the culturing of microbes on resistant starch or the harvesting of such microbes. McNaught discloses the use of resistant starch in foods and industrial products and does nothing to make up for the deficiencies o the other references. No rationale is provided why these unrelated references should be combined other than the fact that each reference lacks one or more limitations of the claims. Moreover, it is entirely unclear what the result would be if the four disparate references were combined. In any event, not one of these references discloses or teaches culturing microbes on a media containing resistant starch and harvesting those microbes.

As detailed in the Declaration of Ian L. Brown submitted February 22, 2005 (Appendix B), Masuda relates to symbiotic combinations of bacteria and does not disclose microbes which have been cultured on resistant starch. Moreover, the Examiner acknowledges that Masuda lacks "disclosure about the use of resistant starch in the total product".

Brown '050 does not cure this deficiency of Masuda because it is directed to probiotics and does not teach the use of resistant starch in a culture medium from which

microbes are to be harvested. Instead, Brown '050 teaches the combination of resistant starch with microbes previously harvested from a different culture medium.

The Examiner applied Brown, Food Australia as teaching that the addition of resistant starches into probiotic microbial products improves "robustness and viability of probiotics in the GI tract" and that high amylose resistant starch "enhances bacterial survival and stress resistance". This protective effect is dependent upon the presence of resistant starch and is not the Applicants' invention!

The microbes of Brown, Food Australia are ingested in combination with a resistant starch which physically protects the microbes in the gut. This is explained on page 608 which states that the "survival of the *Bifidobacterium* in the presence of the high amylose maize starch appears to be linked to the observation that some types of bacteria adhere to the surface of the starch granules" and that "these bacteria often demonstrated enhanced resistance to hostile conditions." This differs from the microbes of the present invention which have enhanced resistance whether or not resistant starch is present! This protection is the result of a change in the microbe itself from being cultured on resistant starch, and is not a physical protection.

While McNaught is relied upon by the Examiner to demonstrate that certain resistant starches were known in the prior art, it does not cure the remaining deficiencies mentioned above, particularly that microbes cultured on resistant starch and harvested there from have improved survival/recovery properties. For these reasons, the rejections of claims 77-153 should be reversed.

D. THE REJECTION UNDER THE JUDICIALLY CREATED DOCTRINE OF OBVIOUS-TYPE DOUBLE PATENTING OVER BROWN '350 SHOULD BE REVERSED BECAUSE THE PROBIOTIC BROWN '350 ORGANISMS WERE NOT CULTURED ON RESISTANT STARCH AND DO NOT HAVE THE IMPROVED SURVIVAL/RECOVERY PROPERTIES OF MICROBES WHICH HAVE BEEN

Finally, the obviousness-type double patenting rejection of claims 77-153 over claims 1-12 of Brown, U.S. Patent No. 6,221,350 ("Brown '350") should also be reversed because the microbes of Brown '350 are not cultured on media containing resistant starch and as a result do not have the improved survival/recovery properties of the claimed microbes.

While the microbes of the invention and those of Brown '350 can belong to the same species and are able to use resistant starch as a nutritional source they are not the same. This is because the capability of using resistant starch as a nutritional source is not the same as the result of having been cultured on and harvested from resistant starch. Further, there is no disclosure in Brown '350 that the microbes are harvested.

The double patenting rejection reflects a misunderstanding of the difference between the invention and of probiotic compositions. Probiotics are dietary supplements to be consumed by a user that contain beneficial bacteria in combination with a carrier such as starch. The probiotic compositions of Brown '350 comprise the combination of microbes and resistant starch but the microbes of the reference were not the products of culturing on a resistant starch containing media. While the improved microbes of the present invention may now optionally be used to replace the conventional microbes to produce the probiotic compositions of Brown '350, that reference did not disclose the improved microbial products of the invention. Moreover, there is no teaching in Brown '350 suggesting the advantages of culturing microbes on resistant starch that would motivate one of ordinary skill to so modify Brown '350.

Accordingly, the obviousness type double patenting rejection should be reversed and each of claims 77-153 should be allowed.

VIII. CLAIMS

A copy of the claims involved in the present appeal is attached hereto as Appendix A. As indicated above, the claims in Appendix A do include the amendments filed by Applicant on October 22, 2003, and do not include the amendment(s) filed on August 1, 2005.

IX. EVIDENCE

The Declaration of Ian L. Brown Under 37 C.F.R. §1.132 filed on February 25, 2005 and entered by the Examiner on page 9 of the Office Action dated May 31, 2005 is submitted herewith at Appendix B.

Copies of each reference relied upon by the Examiner in his rejections are also submitted at Appendix C.

X. RELATED PROCEEDINGS

No related proceedings are referenced in II. above, or copies of decisions in related proceedings are not provided, hence no Appendix is included.

Dated: February 28, 2006

Respectfully submitted,

By 

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APPENDIX A

Claims Involved in the Appeal of Application Serial No. 09/889,085

Listing of the Claims:

1.-19. (Cancelled)

20. (Withdrawn) A process of preparing a microbial preparation having an increased survival/recovery rate in a product, the process comprising growing or culturing microbes in a media based on or containing resistant starch in a manner such that when subsequently incorporated in a product the survival/recovery rate of the harvested microbes is increased as compared with the same microbes grown or cultured in a media without resistant starch, and harvesting the cultured microbes having an increased survival/recovery rate.

21. (Withdrawn) The process according to claim 20 wherein the product is selected from the group consisting of a food, feed, nutraceutical, pharmaceutical, biocontrol, and bioremediation product.

22. (Withdrawn) The process according to claim 20 wherein the resistant starch is type RS1, RS2, RS3 or RS4.

23. (Withdrawn) The process according to claim 22 wherein the resistant starch is derived from starch selected from the group consisting of maize, rice, barley, wheat, legumes, potatoes, and bananas.

24. (Withdrawn) The process according to claim 23 wherein the resistant starch is derived from a starch having an amylose content of at least 40% (w/w).

25. (Withdrawn) The process according to claim 24 wherein the resistant starch is derived from maize starch.

26. (Withdrawn) The process according to claim 25 wherein the maize starch having an amylose content of at least 70% (w/w).

27. (Withdrawn) The process according to claim 25 wherein the maize starch having an amylose content of at least 80% (w/w).

28. (Withdrawn) The process according to claim 25 wherein the maize starch having an amylose content of at least 90% (w/w).

29. (Withdrawn) The process according to claim 23 wherein the starch is chemically, physically, and/or enzymically treated or modified.

30. (Withdrawn) The process according to claim 29 wherein the chemical modification is selected from the group consisting of oxidation, cross-bonding, etherification, esterification, acidification, dextrinisation, and mixtures thereof.

31. (Withdrawn) The process according to claim 29 wherein the physical treatment is heat-moisture treatment to enhance or increase the resistant starch content of the starch.

32. (Withdrawn) The process according to claim 29 wherein the treatment is by solvent extraction to remove fats and/or minerals from the starch.

33. (Withdrawn) The process according to claim 20 wherein the resistant starch is used in the media at a concentration of 0.01 to 10% (w/w).

34. (Withdrawn) The process according to claim 33 wherein the resistant starch is used in the media at 0.1 to 5% (w/w).

35. (Withdrawn) The process according to claim 33 wherein the resistant starch is used in the media at 1% (w/w).

36. (Withdrawn) The process according to claim 20 wherein in use the microbes are unaffected by stresses including aeration, sheer, freeze drying, freezing, drying including

high, medium and low water activity, elevated temperatures, low temperatures, pressure and pressure fluctuations, low pH, high pH, bile acids, moisture, high osmolarity, low osmolarity, high salt, or combinations thereof.

37. (Withdrawn) The process according to claim 20 wherein the microbial preparation is a probiotic, a starter culture, a biocontrol or bioremediation product.

38. (Withdrawn) The process according to claim 37 wherein the microbes are probiotic microorganisms from the genera selected from the group of consisting of Saccharomyces, Bifidobacterium, Bacteroides, Clostridium, Fusobacterium, Propionibacterium, Streptococcus, Enterococcus, Lactococcus, Staphylococcus, Peptostreptococcus, and Lactobacillus.

39. (Withdrawn) The process according to claim 37 wherein the microbes are starter cultures selected from the group consisting of lactic acid bacteria lactic acid bacteria including lactobacillus, lactococcus and streptococcus, leuconostoc, and yeasts.

40. (Withdrawn) The process according to claim 37 wherein the microbes are suitable for use in biocontrol or bioremediation being selected from the group consisting of bifidobacteria, acidophilus, fungi, Bacillus species, pseudomonads and Alcaligenes.

41. (Previously Presented) A microbial preparation having an increased survival/recovery rate in a product prepared by the process comprising growing or culturing microbes in a media based on or containing resistant starch in a manner such that when subsequently incorporated in a product the survival/recovery rate of the harvested microbes is increased as compared with the same microbes grown or cultured in a media without resistant starch, and harvesting the cultured microbes having an increased survival/recovery rate.

42-62. (Cancelled)

63. (Withdrawn) Use of resistant starch in a microbial culture media to produce microbes which when used subsequently in a product after being harvested from the media, have an increased survival/recovery rate as compared with the same microbes grown or cultured in a media without resistant starch.

64. (Withdrawn) The use according to claim 63 wherein the product is selected from the group consisting of a food, feed, nutraceutical, pharmaceutical, biocontrol, and bioremediation product.

65. (Withdrawn) The use according to claim 64 wherein the resistant starch is type RS1, RS2, RS3 or RS4.

66. (Withdrawn) The use according to claim 65 wherein the resistant starch is derived from starch selected from the group consisting of maize, rice, barley, wheat, legumes, potatoes, and bananas.

67. (Withdrawn) The use according to claim 66 wherein the resistant starch is derived from a starch having an amylose content of at least 40% (w/w).

68. (Withdrawn) The use according to claim 67 wherein the resistant starch is derived from maize starch.

69. (Withdrawn) The use according to claim 68 wherein the maize starch having an amylose content of at least 70% (w/w).

70. (Withdrawn) The use according to claim 68 wherein the maize starch having an amylose content of at least 80% (w/w).

71. (Withdrawn) The use according to claim 68 wherein the maize starch having an amylose content of at least 90% (w/w).

72. (Withdrawn) The use according to claim 66 wherein the starch is chemically, physically, and/or enzymically treated or modified.

73. (Withdrawn) The use according to claim 72 wherein the chemical modification is selected from the group consisting of oxidation, cross-bonding, etherification, esterification, acidification, dextrinisation, and mixtures thereof.

74. (Withdrawn) The use according to claim 72 wherein the physical treatment is heat-moisture treatment to enhance or increase the resistant starch content of the starch.

75. (Withdrawn) The use according to claim 72 wherein the treatment is by solvent extraction to remove fats and/or minerals from the starch.

76. (Previously Presented) A product containing microbes having an increased survival/recovery rate, the product including a microbial preparation according to claim 41.

77. (Previously Presented) A microbial preparation comprising harvested microbes which have been grown or cultured in a media based on or containing resistant starch in a manner such that when subsequently incorporated in a product, the survival/recovery rate of the harvested microbes is increased as compared with the same microbes grown or cultured in a media without resistant starch, the product being selected from the group consisting of a food, feed, nutraceutical, pharmaceutical, biocontrol, and bioremediation product, wherein the resistant starch is type RS1, RS3, or RS4.

78. (Previously Presented) The microbial preparation according to claim 77 further comprising resistant starch.

79. (Previously Presented) A microbial preparation comprising harvested microbes which have been grown or cultured in a media based on or containing resistant starch in a manner such that when subsequently incorporated in a product, the survival/recovery rate of the harvested microbes is increased as compared with the same microbes grown or cultured in a media without resistant starch, the product being selected

from the group consisting of a food, feed, nutraceutical, pharmaceutical, biocontrol, and bioremediation product, wherein the resistant starch is derived from starch selected from the group consisting of rice, barley, wheat, legumes, bananas, and combinations thereof.

80. (Previously Presented) The microbial preparation according to claim 79 further comprising resistant starch.

81. (Previously Presented) A microbial preparation comprising harvested microbes which have been grown or cultured in a media based on or containing resistant starch in a manner such that when subsequently incorporated in a product, the survival/recovery rate of the harvested microbes is increased as compared with the same microbes grown or cultured in a media without resistant starch, the product being selected from the group consisting of a food, feed, nutraceutical, pharmaceutical, biocontrol, and bioremediation product, wherein the resistant starch is derived from a starch having an amylose content of at least 40% (w/w).

82. (Previously Presented) The microbial preparation according to claim 81 further comprising resistant starch.

83. (Previously Presented) The microbial preparation according to claim 82 wherein the resistant starch is derived from starch selected from the group consisting of maize, rice, barley, wheat, legumes, potatoes, and bananas, and combinations thereof.

84. (Previously Presented) The microbial preparation according to claim 83 wherein the resistant starch is derived from maize starch.

85. (Previously Presented) The microbial preparation according to claim 84 wherein the maize starch having an amylose content of at least 70% (w/w).

86. (Previously Presented) The microbial preparation according to claim 85 wherein the maize starch having an amylose content of at least 80% (w/w).

87. (Previously Presented) The microbial preparation according to claim 86 wherein the maize starch having an amylose content of at least 90% (w/w).

88. (Previously Presented) A microbial preparation comprising harvested microbes which have been grown or cultured in a media based on or containing resistant starch in a manner such that when subsequently incorporated in a product, the survival/recovery rate of the harvested microbes is increased as compared with the same microbes grown or cultured in a media without resistant starch, the product being selected from the group consisting of a food, feed, nutraceutical, pharmaceutical, biocontrol, and bioremediation product, wherein the starch is chemically, physically, and/or enzymically treated or modified.

89. (Previously Presented) The microbial preparation according to claim 88 further comprising resistant starch.

90. (Previously Presented) The microbial preparation according to claim 88 wherein the resistant starch is derived from starch selected from the group consisting of maize, rice, barley, wheat, legumes, potatoes, and bananas, and combinations thereof.

91. (Previously Presented) The microbial preparation according to claim 88 wherein the chemical modification is selected from the group consisting of oxidation, cross-bonding, etherification, esterification, acidification, dextrinisation, and mixtures thereof.

92. (Previously Presented) The microbial preparation according to claim 88 wherein the physical treatment is heat-moisture treatment to enhance or increase the resistant starch content of the starch.

93. (Previously Presented) The microbial preparation according to claim 88 wherein the treatment is by solvent extraction to remove fats and/or minerals from the starch.

94. (Previously Presented) The microbial preparation according to claim 77 wherein when incorporated in a product, in use the microbes are substantially resistant to stresses including selected from the group consisting of aeration, sheer, freeze drying, freezing, drying including high, medium and low water activity, elevated temperatures, low temperatures, pressure and pressure fluctuations, low pH, high pH, bile acids, moisture, high osmolarity, low osmolarity, high salt, or and combinations thereof.

95. (Previously Presented) The microbial preparation according to claim 77 wherein the microbes are being a probiotic, a starter culture, or a biocontrol or bioremediation product.

96. (Previously Presented) The microbial preparation according to claim 95 wherein the microbes are probiotic microorganisms from the genera selected from the group of consisting of Saccharomyces, Bifidobacterium, Bacteroides, Clostridium, Fusobacterium, Propionibacterium, Streptococcus, Enterococcus, Lactococcus, Staphylococcus, Peptostreptococcus, Lactobacillus, and combinations thereof.

97. (Previously Presented) The microbial preparation according to claim 95 wherein the microbes are starter cultures selected from the group consisting of yeasts, lactic acid bacteria, and combinations thereof.

98. (Previously Presented) The microbial preparation according to claim 97 wherein the lactic acid bacteria are selected from the group consisting of Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, and combinations thereof.

99. (Previously Presented) The microbial preparation according to claim 95 wherein the microbes are suitable for use in biocontrol or bioremediation being selected from the group consisting of Bifidobacterium, Lactobacillus, fungi, Bacillus, Pseudomonas Alcaligenes, and combinations thereof.

100. (Previously Presented) A product comprising a microbial preparation according to claim 77.

101. (Previously Presented) The product according to claim 100 selected from the group consisting of fluid-based food products, water-based fluids, cereal and plant-based food products, solid-based food products, tablets, food additives, health supplements, pharmaceutical preparations, and combinations thereof.

102. (Previously Presented) The product according to claim 101 wherein the fluid-based food products comprise milk-based products where the edible ingredient is one or more milk-based ingredients comprising whole milk, milk solids, milk fat, cream, non-fat dried milk, any other component or derivative from milk suitable for use in milk-based products.

103. (Previously Presented) The product according to claim 101 wherein the solid-based food products are selected from the group consisting of snack bars, breakfast cereals, bread, confectionary, extruded food products, muesli bars, buns, biscuits, feed pellets, coated food products, and combinations thereof.

104. (Previously Presented) The product according to claim 100 being a food product suitable to contain and deliver probiotic microorganisms.

105. (Previously Presented) The food product according to claim 104 selected from the group consisting of food stuffs, fruit beverages, water ices, confectionary, coatings or covertures, yoghurts, yoghurt drinks, unfermented drinks, flavoured milk drinks, modified milk drinks, ice-creams, dairy desserts, and combinations thereof.

106. (Previously Presented) The product according to claim 102 further comprising resistant starch.

107. (Previously Presented) The product according to claim 106 wherein the resistant starch is added at a concentration of 0.1 to 90% (w/w) total product.

108. (Previously Presented) The product according to claim 107 wherein the resistant starch is added at a concentration of about 10% (w/w) total product.

109. (Previously Presented) The microbial preparation according to claim 79 wherein when incorporated in a product, in use the microbes are substantially resistant to stresses including selected from the group consisting of aeration, sheer, freeze drying, freezing, drying including high, medium and low water activity, elevated temperatures, low temperatures, pressure and pressure fluctuations, low pH, high pH, bile acids, moisture, high osmolarity, low osmolarity, high salt, or and combinations thereof.

110. (Previously Presented) The microbial preparation according to claim 79 wherein the microbes are being a probiotic, a starter culture, or a biocontrol or bioremediation product.

111. (Previously Presented) The microbial preparation according to claim 110 wherein the microbes are probiotic microorganisms from the genera selected from the group of consisting of Saccharomyces, Bifidobacterium, Bacteroides, Clostridium, Fusobacterium, Propionibacterium, Streptococcus, Enterococcus, Lactococcus, Staphylococcus, Peptostreptococcus, Lactobacillus, and combinations thereof.

112. (Previously Presented) The microbial preparation according to claim 110 wherein the microbes are starter cultures selected from the group consisting of yeasts, lactic acid bacteria, and combinations thereof.

113. (Previously Presented) The microbial preparation according to claim 112 wherein the lactic acid bacteria are selected from the group consisting of Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, and combinations thereof.

114. (Previously Presented) The microbial preparation according to claim 110 wherein the microbes are suitable for use in biocontrol or bioremediation being selected from

the group consisting of Bifidobacterium, Lactobacillus, fungi, Bacillus, Pseudomonas Alcaligenes, and combinations thereof.

115. (Previously Presented) A product comprising a microbial preparation according to claim 79.

116. (Previously Presented) The product according to claim 115 selected form the group consisting of fluid-based food products, water-based fluids, cereal and plant-based food products, solid-based food products, tablets, food additives, health supplements, pharmaceutical preparations and combinations thereof.

117. (Previously Presented) The product according to claim 116 wherein the fluid-based food products comprise milk-based products where the edible ingredient is one or more milk-based ingredients comprising whole milk, milk solids, milk fat, cream, non-fat dried milk, any other component or derivative from milk suitable for use in milk-based products.

118. (Previously Presented) The product according to claim 116 wherein the solid-based food products are selected from the group consisting of snack bars, breakfast cereals, bread, confectionary, extruded food products, muesli bars, buns, biscuits, feed pellets, coated food products, and combinations thereof.

119. (Previously Presented) The product according to claim 115 being a food product suitable to contain and deliver probiotic microorganisms.

120. (Previously Presented) The food product according to claim 119 selected from the group consisting of food stuffs, fruit beverages, water ices, confectionary, coatings or covertures, yoghurts, yoghurt drinks, unfermented drinks, flavoured milk drinks, modified milk drinks, ice-creams, dairy desserts, and combinations thereof.

121. (Previously Presented) The product according to claim 115 further comprising resistant starch.

122. (Previously Presented) The product according to claim 121 wherein the resistant starch is added at a concentration of 0.1 to 90% (w/w) total product.

123. (Previously Presented) The product according to claim 122 wherein the resistant starch is added at a concentration of about 10% (w/w) total product.

124. (Previously Presented) The microbial preparation according to claim 81 wherein when incorporated in a product, in use the microbes are substantially resistant to stresses including selected from the group consisting of aeration, sheer, freeze drying, freezing, drying including high, medium and low water activity, elevated temperatures, low temperatures, pressure and pressure fluctuations, low pH, high pH, bile acids, moisture, high osmolarity, low osmolarity, high salt, or and combinations thereof.

125. (Previously Presented) The microbial preparation according to claim 81 wherein the microbes are being a probiotic, a starter culture, or a biocontrol or bioremediation product.

126. (Previously Presented) The microbial preparation according to claim 125 wherein the microbes are probiotic microorganisms from the genera selected from the group of consisting of Saccharomyces, Bifidobacterium, Bacteroides, Clostridium, Fusobacterium, Propionibacterium, Streptococcus, Enterococcus, Lactococcus, Staphylococcus, Peptostreptococcus, Lactobacillus, and combinations thereof.

127. (Previously Presented) The microbial preparation according to claim 125 wherein the microbes are starter cultures selected from the group consisting of yeasts, lactic acid bacteria, and combinations thereof.

128. (Previously Presented) The microbial preparation according to claim 126 wherein the lactic acid bacteria are selected from the group consisting of Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, and combinations thereof.

129. (Previously Presented) The microbial preparation according to claim 125 wherein the microbes are suitable for use in biocontrol or bioremediation being selected from the group consisting of Bifidobacterium, Lactobacillus, fungi, Bacillus, Pseudomonas Alcaligenes, and combinations thereof.

130. (Previously Presented) A product comprising a microbial preparation according to claim 81.

131. (Previously Presented) The product according to claim 130 selected form the group consisting of fluid-based food products, water-based fluids, cereal and plant-based food products, solid-based food products, tablets, food additives, health supplements, pharmaceutical preparations, and combinations thereof.

132. (Previously Presented) The product according to claim 131 wherein the fluid-based food products comprise milk-based products where the edible ingredient is one or more milk-based ingredients comprising whole milk, milk solids, milk fat, cream, non-fat dried milk, any other component or derivative from milk suitable for use in milk-based products.

133. (Previously Presented) The product according to claim 131 wherein the solid-based food products are selected from the group consisting of snack bars, breakfast cereals, bread, confectionary, extruded food products, muesli bars, buns, biscuits, feed pellets, coated food products, and combinations thereof.

134. (Previously Presented) The product according to claim 130 being a food product suitable to contain and deliver probiotic microorganisms.

135. (Previously Presented) The food product according to claim 134 selected from the group consisting of food stuffs, fruit beverages, water ices, confectionary, coatings or covertures, yoghurts, yoghurt drinks, unfermented drinks, flavoured milk drinks, modified milk drinks, ice-creams, dairy desserts, and combinations thereof.

136. (Previously Presented) The product according to claim 130 further comprising resistant starch.

137. (Previously Presented) The product according to claim 136 wherein the resistant starch is added at a concentration of 0.1 to 90% (w/w) total product.

138. (Previously Presented) The product according to claim 137 wherein the resistant starch is added at a concentration of about 10% (w/w) total product.

139. (Previously Presented) The microbial preparation according to claim 88 wherein when incorporated in a product, in use the microbes are substantially resistant to stresses including selected from the group consisting of aeration, sheer, freeze drying, freezing, drying including high, medium and low water activity, elevated temperatures, low temperatures, pressure and pressure fluctuations, low pH, high pH, bile acids, moisture, high osmolarity, low osmolarity, high salt, or and combinations thereof.

140. (Previously Presented) The microbial preparation according to claim 88 wherein the microbes are being a probiotic, a starter culture, or a biocontrol or bioremediation product.

141. (Previously Presented) The microbial preparation according to claim 140 wherein the microbes are probiotic microorganisms from the genera selected from the group of consisting of Saccharomyces, Bifidobacterium, Bacteroides, Clostridium, Fusobacterium, Propionibacterium, Streptococcus, Enterococcus, Lactococcus, Staphylococcus, Peptostreptococcus, Lactobacillus, and combinations thereof.

142. (Previously Presented) The microbial preparation according to claim 140 wherein the microbes are starter cultures selected from the group consisting of yeasts, lactic acid bacteria, and combinations thereof.

143. (Previously Presented) The microbial preparation according to claim 142 wherein the lactic acid bacteria are selected from the group consisting of Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, and combinations thereof.

144. (Previously Presented) The microbial preparation according to claim 140 wherein the microbes are suitable for use in biocontrol or bioremediation being selected from the group consisting of Bifidobacterium, Lactobacillus, fungi, Bacillus, Pseudomonas Alcaligenes, and combinations thereof.

145. (Previously Presented) A product comprising a microbial preparation according to claim 88.

146. (Previously Presented) The product according to claim 145 selected form the group consisting of fluid-based food products, water-based fluids, cereal and plant-based food products, solid-based food products, tablets, food additives, health supplements, pharmaceutical preparations, and combinations thereof.

147. (Previously Presented) The product according to claim 146 wherein the fluid-based food products comprise milk-based products where the edible ingredient is one or more milk-based ingredients comprising whole milk, milk solids, milk fat, cream, non-fat dried milk, any other component or derivative from milk suitable for use in milk-based products.

148. (Previously Presented) The product according to claim 146 wherein the solid-based food products are selected from the group consisting of snack bars, breakfast cereals, bread, confectionary, extruded food products, muesli bars, buns, biscuits, feed pellets, coated food products, and combinations thereof.

149. (Previously Presented) The product according to claim 145 being a food product suitable to contain and deliver probiotic microorganisms.

150. (Previously Presented) The food product according to claim 149 selected from the group consisting of food stuffs, fruit beverages, water ices, confectionary, coatings or covertures, yoghurts, yoghurt drinks, unfermented drinks, flavoured milk drinks, modified milk drinks, ice-creams, dairy desserts, and combinations thereof.

151. (Previously Presented) The product according to claim 145 further comprising resistant starch.

152. (Previously Presented) The product according to claim 151 wherein the resistant starch is added at a concentration of 0.1 to 90% (w/w) total product.

153. (Previously Presented) The product according to claim 152 wherein the resistant starch is added at a concentration of about 10% (w/w) total product.

APPENDIX B

Declaration of Ian L. Brown Submitted February 22, 2005

Docket No.: 28053/38258
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	I hereby certify that this paper is being deposited with the U.S. Postal Service as First Class Mail in an envelope, postage prepaid, addressed to the Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on this date:
Patricia L. CONWAY et al.	
Serial No.: 09/889,085	
Filed: January 9, 2002	**February 22, 2005**
For: IMPROVED MICROBIAL PREPARATIONS	Jeffrey S. Sharp Registration No. 31,879 Attorney for Applicants
Group Art Unit: 1651	
Examiner: Vera Afremova	

DECLARATION OF IAN L. BROWN
PURSUANT TO 37 CFR § 1.132

I, Ian L. Brown, a resident of the United States, residing at 24 Hancock Court, Basking Ridge, New Jersey 07920, hereby declare that:

1. I am a co-inventor of the invention disclosed and claimed in the above-identified patent application, U.S. Patent Application Serial No. 09/889,085 ("the patent application"), filed January 9, 2002. As such, I am thoroughly familiar with the patent application as originally filed and amended.

2. My qualifications and technical experience are set out in my *curriculum vitae*, a copy of which is attached as Appendix A.

3. I have read and understand the official action from the U.S. Patent and Trademark Office (the "Patent Office") dated August 24, 2004 (the "Office Action"), which was issued in connection with U.S. Patent Application Serial No. 09/889,085. I also have reviewed and understand the patents and publications cited by the Examiner in the Office Action. I make this Declaration to provide information known to me that may be relevant to various claim rejections in the Office Action.

4. The Examiner raised an issue regarding the disclosure of Masuda U.S. Patent No. 5,143,845 in which she suggested that the products of Example 2 comprising microbes incubated in the presence of potato starch would inherently anticipate the applicants' claims. This interpretation is incorrect because Masuda makes clear (and those of skill in the art would recognize) that the potato starch compositions of Masuda are cooked in a manner such that no resistant starch would remain.

5. Specifically, Masuda is directed to a method of preparing microbial cultures on starch media and any of the materials used would be sterilized before their inclusion in the microbial cultures in order to prevent contamination with other cultures. See Example 2, col. 2, lines 57-58 which describes autoclaving the basal media.

6. It is well known that potato starch is resistant in its uncooked state but is digestible (non-resistant) after cooking. Specifically, Rendleman, Biotechnol. Appl. Biochem. 31:171-178 (2000) (Appendix B) describes that uncooked potato starch was only 10.9% degraded to G1 to G7 residues after 8 hours in the presence of alpha-amylase. (Table 2) In contrast, potato starch which had been cooked at 100°C for 30 minutes was 88.6% degraded to G1 to G7 residues. (Table 3) Thus, the cooking process of Masuda destroyed the potato starch granule so that the resultant material is no longer RS2 (uncooked starch). Because the residues referred to are small fragments of seven glucose residues or less (the majority contain two (2) glucose units) they are too small to form into RS3 (retrograded starch). See also, Woodruff and Nicoli Starch gels, Cereal Chemistry 8:243 (1931) (Appendix C) which teaches the gelatinization temperature of potato starch to be only 69-70°C. Finally, Raben et al., J. Clin. Nutr. 60: 544-551 (1994) (Appendix D) states that raw potato starch has a resistant starch content of about 54% while pregelatinized potato starch comprises 0% resistant starch. The pregelatinized potato starch is simply potato starch that has been cooked in water to completely gelatinize the starch and is dried.

7. Because standard autoclave conditions are the use of 121°C at 15 psig for a minimum of 30 minutes (See Effective Use of Autoclaves: Safety Net #26 - UC Davis Environmental Health and Safety - 2-1493) (Appendix E) it is clear that the potato starch components used in the Masuda examples are substantially free of resistant starch.

8. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or document or any patent which may issue thereon.

Ian D. Brown, Ph.D.

Date: 9th February 2005

APPENDIX C
References Cited by the Examiner

Masuda, U.S. Patent 5,143,845.

Brown et al., U.S. Patent 6,060,050 ("Brown '050")

McNaught et al., U.S. Patent 5,714,600.

Brown et al., *Food Australia 50(12)*, 603-610, (Dec. 1998) ("Brown, Food Australia")

Brown et al., U.S. Patent 6,221,350 ("Brown '350")

IAN LEWIS BROWN

Present Position: Senior Director – Nutrition, National Starch and Chemical Company.
10 Finderne Avenue,
Bridgewater, 08807 New Jersey USA.
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Education:

High School Diploma (1976) Forest Park, Ohio, USA.
Higher School Certificate (1976) NSW, Australia.

Bachelor of Science. (1981) The University of New England, Australia.

Diploma of Education. (1981) The University of New England.

Diploma of Business Studies. (1989) The University of New England.

Master of Science (Biochemistry). (1995) The University of New England.
Thesis entitled "The structure of Australian maize starch"

Doctor of Philosophy (Applied Bioscience, Graduate School of Agriculture). (1999) The University of Hokkaido, Japan.
Thesis entitled "The development and application of high amylose maize starches for food, nutritional benefit and public health".

Appointments:

2004 - Visiting Professor, School of Medicine
University of Colorado Health Sciences Center
[Colorado University, USA]

2000 - Professorial Fellow, University of Wollongong
[ARC Smart Foods Key Centre & Faculty of Health & Behavioural Sciences, University of Wollongong, Australia]

2000 - Member of Advisory Board for Australian Research Council (ARC) Key Centre for Smart Foods.
[University of Wollongong, Australia]

2003 - Senior Director - Nutrition, National Starch and Chemical Company.

Professional Associations:

1985 - 2001 Foundation Member of the Australian Biotechnology Assoc.
1986 - Member of the Royal Australian Chemical Institute.
1986 - Professional Member of the Australian Institute of Food Science & Technology.
1986 - Professional Member of the Institute of Food Technologists USA.
1990 - Associate Fellow of the Australian Institute of Management.
1994 - Member of the Nutrition Society of Australia.
2000 - Professional Member of the American Association of Cereal Chemists.

Awards:

1975-76 American Field Service Scholarship to study in the USA for 12 months.

1977-80 NSW Department of Education Teaching Scholarship.

1994 RACI [Royal Australian Chemical Institute] **Cereal Chemistry Division Poster Paper Award – Best Visual Presentation.**

1995 RACI [Royal Australian Chemical Institute] **Cereal Chemistry Division Poster Paper Award – Best Poster Contributed by a Commercial Industry Laboratory.**

1995 AIFST [Australian Institute of Food Science & Technology] **Food Industry Innovation Award.**

1999 AIFST [Australian Institute of Food Science & Technology] **The Jack Kefford Award for the Best Paper Published in "Food Australia" in 1998.**

Government: **1998 – 1999.** Member of the external technical advisory committee that assisted in the preparation of the Australian & New Zealand Food Authority Full Assessment Report. Proposal P177. Derivation of Energy Factors. February 1999 [Development of Joint Australian New Zealand Food Standards as part of the process of the Review of the Food Standards Code].

2000 - 2001. Member of the Expert Working Group on Dietary Fibre for the Australian & New Zealand Food Authority.

Patents: Co-inventor of 10 USA patents (more than 32 patents granted internationally in other countries) concerning maize varieties, starch production, resistant starch and dietary fibre utilisation, nutrition, prebiotic functionality of starch and other food ingredients, treatment of human and animal diseases, and the physiological action of starch and other dietary components.

Publications: Published 27 papers and one book chapter in a variety of international scientific journals on topics concerning cereal science, starch chemistry, food engineering, nutrition and microbiology.

Some 38 other publications have been authored in association with conferences and symposia in Australia and overseas.

Provided educational segments for courses sponsored by the Bread Research Institute, Royal Australian Chemical Institute and University of Wollongong.

Invited & Plenary Presentations:

Australia: Invited to present oral papers at 19 conferences and symposia in Australia.

International: Invited to provide oral papers or chair meetings at 16 international conferences and symposia in Japan, Singapore, Malaysia, New Zealand, USA, Canada, Korea, Ireland and the Philippines.

Committees: Honorary Treasurer for 3rd International Food Hydrocolloids Conference. Sydney. (1996).

Member of the Management Committee of the Cooperative Research Centre for Food Industry Innovation (1996 - 00).

Grants: Successful in obtaining research grants through AIR&DIB and Food into Asia schemes.

Ian Lewis Brown

7 June 2004

Hydrolytic action of α-amylase on high-amylose starch of low molecular mass

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High-amylose starches of low average degree of polymerization (d̄p 61–71), formed as fine granules by interaction of *Bacillus macerans* cyclodextrin glucanotransferase with α-cyclodextrin (CD) at 2–70 °C, are highly insoluble in water and not gelatinizable under normal cooking conditions (100 °C). Samples of CD-derived starches, both cooked and uncooked, were subjected to hydrolysis *in vitro* by human salivary α-amylase at 37 °C under conditions chosen to resemble those in the human intestinal lumen. Released low-molecular-mass saccharides were determined quantitatively by HPLC and the results compared with those from similar studies with natural starches. Among uncooked starches, CD-derived starch showed very low reactivity towards α-amylase, along with potato starch and a high-amylose hybrid corn starch (64% amylose). Cooking greatly enhanced reactivity of natural starches, but only moderately increased reactivity of CD-derived starches. Susceptibility to hydrolysis of cooked starches increased in the following general order: CD-derived starch (≈ 100% amylose) < 100% corn amylose (isolated by the butan-1-ol method) < hybrid high-amylose corn starch (64–66% amylose) < waxy maize starch (99–100% amylopectin) ~ ordinary corn starch (≈ 25% amylose) < potato starch (≈ 25% amylose).

Introduction

Starch is the most extensively used polysaccharide in food preparations and serves as the principal nutrient in the diet of the Western world. Digestion of starch by humans occurs primarily in the small intestine where two separate families of carbohydrases (enzymes that hydrolyse carbohydrates) are found [1]. One family is introduced into the intestinal lumen and is represented by α-amylases (salivary and pancreatic). The other family is immobilized in the brush-border membrane and includes disaccharidases (such as maltase, lactase and sucrase), trisaccharidases and glucoamylase (amyloglucosidase). Glucoamylase liberates glucose specifically from the non-reducing end of malto-oligosaccharides (with greatest affinity for chain lengths of 5–9 glucose units [1]). Pancreatic α-amylase predominates in the small intestine and is located largely in the lumen. α-Amylase specifically catalyses the hydrolysis of α-1,4 glycosidic linkages in starch. Amylose, a linear α-D-glucan, is hydrolysed to maltotriose, maltose and a small amount of free glucose. Hydrolysis of amylopectin, the highly branched form of starch, likewise yields maltotriose, maltose and a small amount of glucose; however, because of the numerous enzyme-resistant branch points, α-limit dextrins (α-1,6-glucosides with 3–6 glucose units) are also formed. Action by α-amylase on maltotriose to produce maltose and glucose is very weak and, therefore, of minor importance in the overall production of maltose and glucose. Maltose appears to be totally resistant to the enzyme. Because only monosaccharides are absorbed through the intestinal wall, maltose must first be hydrolysed to glucose by membrane-bound carbohydrases in the brush border. These same brush-border enzymes contribute to the breakdown of other malto-oligosaccharides. Glucose is absorbed at the rate of 5–20 g/h in the adult duodenum, and at 25–40 g/h in a 45-cm segment of the jejunum.

Although dissolved or gelatinized forms of natural starches react rapidly towards α-amylase, reaction rates of raw or granular forms are much slower and vary according to the source of starch. Leach and Schoch [2] found the following order of increasing resistance of granules towards α-amylase: waxy maize < tapioca < waxy sorghum < sorghum < ordinary corn < wheat < rice < sago < arrowroot < potato < high-amylose corn. At 50 °C, salivary α-amylase hydrolyses granular corn starch at one-eighth of the rate at which it hydrolyses the dissolved form [3]. However, there have been no published studies *in vitro* of enzymic hydrolysis of granular forms of amyloses with a low average degree of polymerization (d̄p ≈ 55–75) under conditions similar to those in the lumen of the human small intestine. Granular forms of this type are readily prepared in good yield from interaction of *Bacillus macerans* cyclodextrin

Key words: α-cyclodextrin, cyclodextrin glucanotransferase, scanning electron microphotography, starch digestion, X-ray diffraction analysis.

Abbreviations used: CD, α-cyclodextrin; CGTase, cyclodextrin glucanotransferase; dp, degree of polymerization; d̄p, average degree of polymerization; SEM, scanning electron microphotograph.

glucanotransferase (CGTase) with α-cyclodextrin (CD) over a wide range of temperatures [4]. Because of the possible usefulness of such amyloses as components of low digestibility in processed foods, a study *in vitro* was initiated in this laboratory to determine the susceptibilities of both cooked and uncooked forms to attack by α-amylase. Conditions of pH, temperature and enzyme concentration were chosen to resemble those conditions in the human small intestine. α-Amylase has optimal activity in near-neutral media (pH 6.9–7.0), similar to the environment in the proximal bowel [5]. Each enzyme molecule requires at least one calcium ion for enzymic activity and to prevent its destruction in the human gut by proteolytic enzymes [6]. Chloride ion is also essential for the action of α-amylases [7], the optimal concentration of Cl^- being ≈ 0.01 M [3]. When human pancreatic α-amylase is not readily available for use in studies of starch hydrolysis, commercially accessible human salivary α-amylase can be used as an adequate substitute. Various investigators have observed a close similarity between properties of the two enzymes [8,9]. Lee has reported that there is no significant difference in the rate of hydrolysis by salivary, pancreatic and mammary α-amylases [10]. Preduodenal hydrolysis by salivary α-amylase is believed to play only a minor role in the overall digestion of starch in adults. Contribution of salivary α-amylase to enzyme activity in the intestinal lumen is small, partly because of its partial inactivation by gastric acid prior to movement of the enzyme into the duodenum. Nevertheless, in normal adults, as much as 11% of the total α-amylase output in postprandial jejunal fluid is salivary [11].

Experimental

Materials

α-Amylase (EC 3.2.1.1) from human saliva (chromatographically purified; 2500 units/mg of solid, as determined by the Bernfeld method [12]) was obtained from Sigma (St. Louis, MO, U.S.A.). According to the Bernfeld method for determining activity, 1 activity unit of this enzyme liberates the equivalent of 1.0 mg of maltose (2.9×10^{-3} mmol) from soluble starch in 3 min at pH 6.9 and 20 °C. However, at the higher temperature of 37 °C the effective activity is 1.3 times greater than that at 20 °C. In this laboratory it was determined that 1 activity unit as determined at 20 °C and pH 6.9 will liberate 3.8×10^{-3} mmol of reducing saccharides [malto-oligosaccharides of degree of polymerization (dp) 2–7 and small amounts of glucose] in 3 min at pH 7.2 and 37 °C. CGTase (EC 2.4.1.19) from *B. macerans* was obtained as an aqueous solution (600 units/ml, pH 7, according to the method of Tilden and Hudson [13]) from Amano International Enzyme Co. (Troy, VA, U.S.A.). CD (8.8% H_2O) was from Anspec/Ohio (Columbus, OH, U.S.A.). Corn amylopectin (11.5% H_2O) and soluble potato starch were from Sigma; waxy-maize corn starch (10.7% H_2O) and ordinary corn starch (10.1% H_2O) were from Cerestar U.S.A. (Hammond, IN, U.S.A.); potato starch (Avebe; 12.0% H_2O), and Hylon VII (64% amylose; 11.1% H_2O), a high-amylose corn starch, were from National Starch and Chemical Corp., Bridgewater, NJ, U.S.A. Corn amylose (≈ 100%; 6.4% H_2O) was prepared at this Center (the National Center for Agricultural Utilization Research, Peoria, IL, U.S.A.) from corn starch by complexation with butan-1-ol. Low-$\bar{d}p$ amyloses were prepared by the conversion of CD with CGTase at temperatures ranging from 2 to 70 °C according to methods described earlier [4]. Typical yields and compositions of these amyloses are presented in Table 1. All starches were stored in a chamber maintained at constant relative humidity (31%). Saccharides, used as standards in HPLC analyses, were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Streptomycin sulphate was from Sigma. For buffering purposes, a stock 0.8 M solution of Tris/HCl, pH 7.2, was prepared. Stock solutions of α-amylase (2000 units/ml of 1 mM $CaCl_2$ solution) were stored in a freezer. Water was distilled and deionized.

Table 1 High-amylose starches of low molecular mass formed by the conversion of CD by CGTase

Data for all conversions other than that at 2 °C were taken from [4]. Amylose analysis was by starch-iodine analysis [14]. High-amylose starch prepared at 2 °C was formed by reaction of CD (22.4 g, anhydrous weight) with CGTase (300 units) for 46 days in aqueous solution (215 ml) at pH 7. Streptomycin sulphate (7 mg) was added to prevent microbial growth. A similar yield was obtained in only 3 days when a much larger amount of CGTase (4800 units) was employed. Yield (wt%) refers to the percentage yield calculated on the basis of the anhydrous weight of the starting material.

Reaction temperature (°C)	Yield (wt%)	Moisture content (%)	Amylose analysis	
			$\bar{d}p$	Amylose (%)
2	54	11.3	61	104
25	53	13.1	63	99
40	65	14.3	65	96
50	68	13.4	69	98
60	78	13.5	69	98
70	72	9.4	71	94

Analytical methods

Low-molecular-mass saccharides (dp 1–7) were determined by HPLC on a DuPont Zorbax NH_2 column (4.6 mm × 250 mm) at 40 °C with acetonitrile/water (13:7, v/v) at 1.0 ml/min and with refractometric detection. The reference standard contained 0.8 mg of each anhydrous carbohydrate/ml of aqueous solution. Calculated yields of saccharides formed by interaction of α-amylase with starch

were based upon the initial amount (in mmol) of anhydroglucose residues in the substrate.

Low-$\bar{d}p$ starches were evaluated for both amylose content and $\bar{d}p$ by spectrophotometric measurement of their amylose–iodine complexes, using the analytical method of Knutson [14] and utilizing the relationships between dp, iodine-binding capacity and wavelength of maximum absorption (λ_{max}) reported by Banks et al. [15]. Amylose contents of natural starches were obtained by similar means. Moisture contents of substrates were determined by heating weighed samples under vacuum at 115 °C for 2 h.

Procedure for enzymic hydrolysis

Into screw-capped culture tubes were placed weighed amounts of starch hydrate and water. A measured volume of stock Tris/HCl buffer (13 μl/ml of reaction mixture) was added to each to impart a Tris concentration of 0.01 M and to ensure that a pH of 6.8–7.0 was maintained throughout enzymic hydrolysis. Cooking, when desired for certain studies, was accomplished at this stage by first immersing the capped tubes in a bath of boiling water (100 °C) for 30 min and then cooling the tubes rapidly to room temperature. After measured volumes of stock α-amylase were added, the tubes were placed in a 37 °C shaker bath for desired periods of time. Reactions were stopped by immersing the tubes in a dry ice/acetone mixture. Reaction mixtures were kept frozen (at or below −30 °C) until time for analysis. No inactivation of α-amylase was necessary. Prior to analysis, each frozen mixture was thawed rapidly and centrifuged briefly at 980 *g*. An aliquot of the supernatant was diluted to an appropriate volume (usually 10 ml/ml of supernatant), and filtered by syringe through a Millipore HV 0.45-μm filter. A sample of filtered solution was immediately injected into an HPLC equipped for saccharide analysis. The time lapse between centrifugation and HPLC injection was approximately 5 min.

Determination of α-amylase activity at 37 °C

Into a capped culture tube was placed 10.0 ml of a 0.08% (w/v) solution of soluble potato starch containing $CaCl_2$ (1 mM), Tris/HCl (5 mM), and sufficient NaOH to give a pH of 7.2. After the tube was placed in a 37 °C water bath, a measured amount of α-amylase (0.4 unit of activity based upon a Bernfeld assay at 20 °C) was introduced. At hourly intervals a 1-ml aliquot was removed, cooled quickly to room temperature, filtered by syringe through a 0.45-μm filter, and subjected immediately to HPLC analysis for saccharides produced during enzymic hydrolysis. The time lapse between removal of sample from the bath and its injection into an HPLC column was 4–6 min. Saccharides of dp >7 were not observed. Graphs of time versus mmol of total saccharides (dp 1–7) and time versus mmol of total

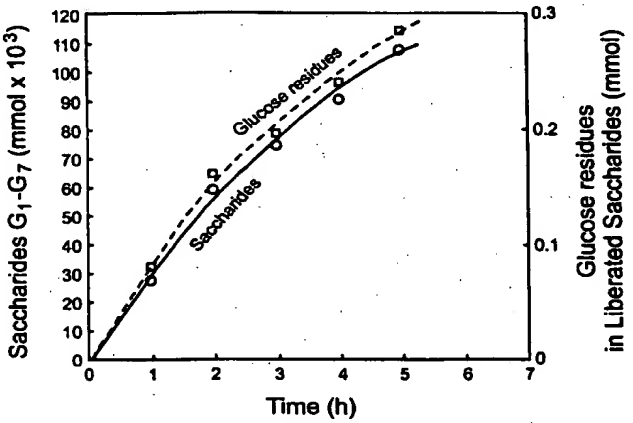

Figure 1 Hydrolysis of soluble potato starch by human salivary α-amylase at 37 °C and pH 7.2

Initial starch solution (10 ml) contained 80 mg of soluble starch (anhydrous basis; ≈ 0.493 mmol of glucose residues) and was 1 mM in $CaCl_2$ and 5 mM in Tris/HCl buffer. Human salivary α-amylase was added to provide an activity of 0.04 unit/ml (based upon a Bernfeld assay at 20 °C [12]). Liberated saccharides (G_1–G_7) were determined by HPLC.

glucose residues in the combined saccharides (Figure 1) permitted a determination of activity not only in terms of total reducing groups produced during the first 3 min of reaction, but also in terms of total glucose residues produced. Liberated glucans appeared to be primarily, if not entirely, straight-chain malto-oligosaccharides, as indicated by their chromatographic retention times. The study revealed that α-amylase activity (in terms of mmol of reducing groups produced) increased by a factor of 1.28 when the temperature was raised from 20 to 37 °C. Thus, at 37 °C and pH 7, a starch solution (10 ml) containing 0.4 unit of α-amylase (based upon a Bernfeld assay at 20 °C) yielded 0.0015 mmol of reducing groups in 3 min. Had the reaction been conducted at 20 °C, only 0.00118 mmol of reducing groups would have been produced. At 37 °C during the first 3 min of reaction, 0.00415 mmol of glucose *residues* was liberated in the form of saccharides of dp 1–7.

Results and discussion

Studies *in vitro* of starch hydrolysis, designed to provide information about starch digestion in humans, necessitated the use of conditions of temperature, pH, reaction time and α-amylase activity associated with digestion in the intestinal lumen. An assumption was made that the average time for passage of food through the small intestine is 8 h. By means of calculations based upon published data, an α-amylase activity of 13 units/ml of reaction mixture was chosen to represent a typical postprandial activity for intestinal contents. In pancreatic secretion, α-amylase activity at 37 °C has been reported to be about 240 units/ml [9]; and the rate of

Table 2 Reaction of salivary α-amylase at 37 °C and pH 7.0 with uncooked starches

Conditions: α-amylase, 13 units/reaction; volume of mixture, 1 ml; starch, 40 mg (anhydrous weight; equivalent to 0.2467 mmol of glucose residues); Tris/HCl buffer, 0.01 M; $CaCl_2$ 1 mM. Abbreviations: G_1, D-glucose; G_2–G_4, straight-chain malto-oligosaccharides of dp 2–4; G_5–G_7, formed only in reactions where the substrate contained an appreciable amount of amylopectin, were probably *branched* glucans of dp 5, 6 and 7, respectively. Their HPLC elution times were significantly greater than the corresponding elution times of maltopentaose, maltohexaose and maltoheptaose. All starches but corn amylose were granular.

Reaction no./starch	Time (h)	Saccharide yields (mmol of G_1 residues × 10^3)								Degradation of starch to G_1–G_7 (%)
		G_1	G_2	G_3	G_4	G_5	G_6	G_7	Combined	
Natural starches										
1. Corn, ordinary (≈ 25% amylose)	2	4.9	35.4	21.3	0.5	0.6	1.5	2.2	66.4	26.9
2. Corn, ordinary (≈ 25% amylose)	8	11.5	74.2	28.5	0	1.8	3.8	3.4	123.2	49.9
3. Corn, ordinary (≈ 25% amylose)	20	16.7	114.8	23.4	0	1.7	3.7	3.3	163.6	66.3
4. Waxy maize (99–100% amylopectin)	8	10.3	88.0	42.6	0	1.7	8.4	3.5	154.5	62.6
5. Hylon VII (hybrid corn; 64% amylose)	8	3.4	19.0	3.0	0	0	0	0	25.4	10.3
6. Amylose, 100% (non-granular; from corn)	8	6.7	62.6	17.7	0	0	0	0	87.0	35.3
7. Potato	8	7.7	15.2	2.0	0	0	2.0	0	26.9	10.9
8. Potato	20	8.9	24.1	0.9	Trace	0.5	1.0	0.5	35.9	14.6
Amyloses, CD-derived, low-$\bar{d}p$										
9. 2 °C product	8	2.8	13.1	1.5	Trace?	0	0	0	17.4	7.1
10. 25 °C product	8	4.5	14.4	2.3	0	0	0	0	21.2	8.6
11. 50 °C product	8	3.9	11.6	2.1	0	0	0	0	17.6	7.1
12. 50 °C product	26	6.8	13.8	0	0	0	0	0	20.6	8.4
13. 70 °C product	8	6.4	23.2	5.4	0	0	0	0	35.0	13.5

flow of this secretion into the duodenum is as high as 1200 ml/day, or 50 ml/h [7]. In the present study, an assumption was made that an average meal is consumed in 1 h and that the ultimate volume of ingested food and drink (including 50 ml of pancreatic juice) is 900 ml. Thus, an upper level of α-amylase activity in the luminal contents would be (240 units/ml × 50 ml of secretion)/900 ml of luminal contents, or 13 units/ml. In several experiments, time and enzyme concentration were varied to provide information about the influence of such variations on the extent of hydrolysis. Salivary α-amylase was employed as a substitute for pancreatic α-amylase in all of the studies.

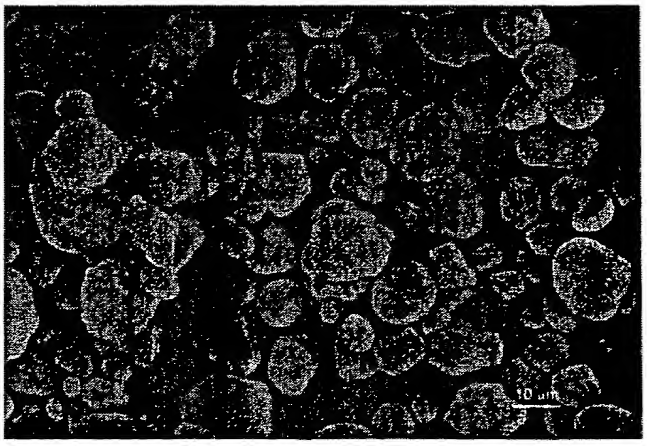

Figure 2 SEM of ordinary corn-starch granules (× 470)

Hydrolysis of uncooked starches

All of the starches used in these studies were granular, with the exception of 100% corn amylose which, because of the method employed in its isolation from ordinary corn starch, was amorphous in appearance under a scanning electron microscope. Table 2 presents data on uncooked natural (native) starches (reactions 1–8) as well as on uncooked low-$\bar{d}p$ amyloses (reactions 9–13) prepared synthetically in this laboratory and described in Table 1. Among the granular natural starches, only Hylon VII and potato starch possessed low susceptibility towards attack by α-amylase. The moderately high susceptibility of 100% corn amylose was probably related, at least in part, to its non-granular structure. X-ray diffraction analysis of this amylose revealed a V pattern, a type normally associated with amylose complexes in which guest molecules are bound within the tunnel-like cavities of starch-chain helices. The complexant butan-1-ol, which had been used in the isolation of amylose from corn starch, was not present in the amylose, since it had been removed by azeotropic distillation at the conclusion of the isolation process. Examination of the amylose by solid NMR did not reveal the presence of any complexant. Yet, the V pattern suggested strongly that although the conditions of azeotropic distillation were sufficient for removal of complexant, they were not sufficient to allow extensive formation of 'crystalline' amylose, a spherulitic form of starch known to possess a B-type structure and to be highly resistant towards α-amylase [16]. An X-ray diffraction pattern of type B was obtained for Hylon VII, whose granules have smooth surfaces that offer considerable resistance to enzymic attack (Table 2; reaction 5). Low-$\bar{d}p$ amylose granules derived from CD at 2–60 °C also have

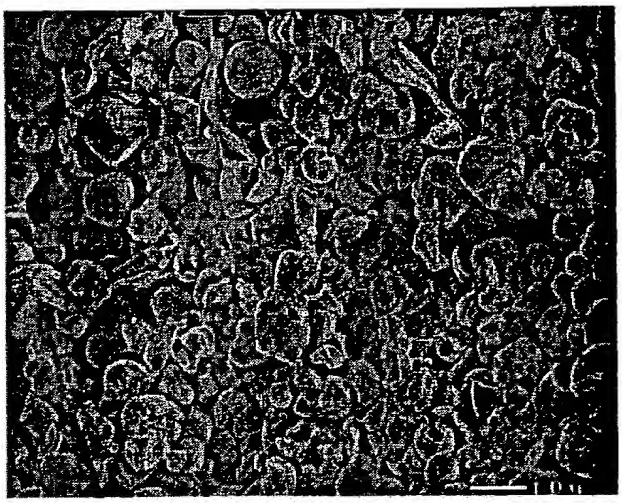

Figure 3 SEM of ordinary corn-starch granules after treatment in aqueous solution with salivary α-amylase at 37 °C (× 500)

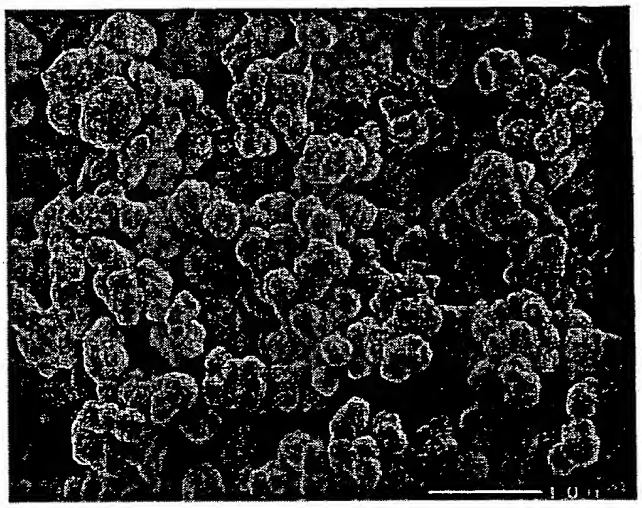

Figure 5 SEM of CD-derived amylose granules prepared at 25 °C (× 1000)

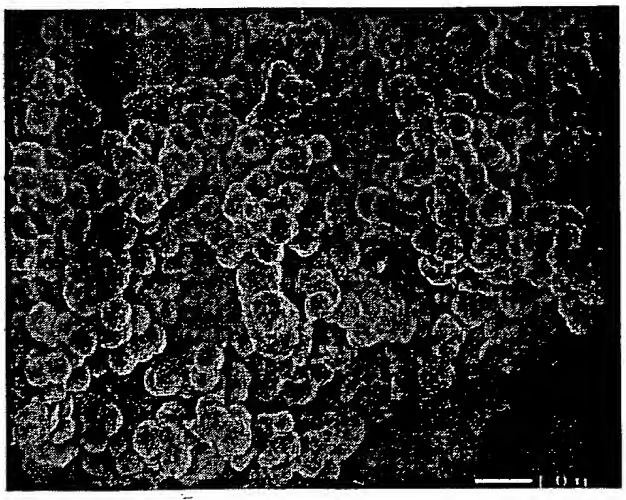

Figure 4 SEM of CD-derived amylose granules prepared at 2 °C (× 500)

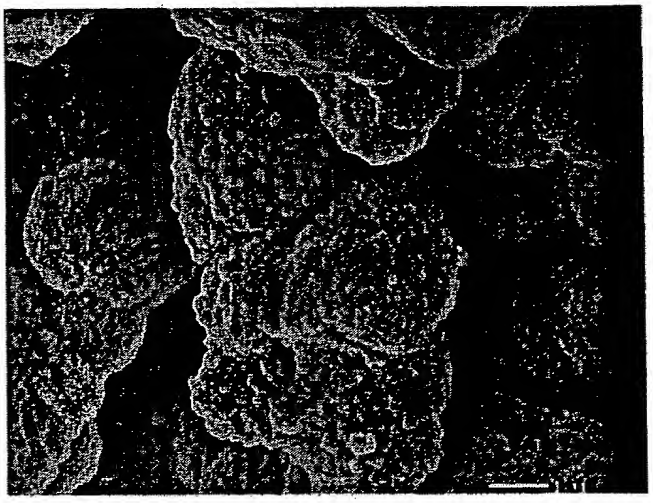

Figure 6 SEM of CD-derived amylose granules prepared at 25 °C (× 5000)

type-B diffraction patterns. Their solubility in water is low and their resistance to α-amylase is similar to that of Hylon VII granules and potato-starch granules.

For the natural starches listed in Table 2, the order of increasing resistance to α-amylase is the same as that found by Leach and Schoch [2]. The positions of low-$\bar{d}p$ amyloses in this series are generally slightly higher than that of potato starch: waxy maize < ordinary corn < corn amylose (isolated from corn starch) < potato ~ Hylon VII < CD-derived amyloses. The relatively high reactivity of waxy maize and ordinary corn starch was expected because of the results of earlier studies by Fannon and co-workers [17,18], who investigated surface pores that occur on granules of corn, sorghum and millet starches, but not on granules of potato, rice, wheat, oat, tapioca, arrowroot and canna. Those investigators proposed that the pores, which appear to be openings that lead to serpentine channels that penetrate into the granule interior, allow access of hydrolysing enzymes and thereby affect the pattern of enzymic attack. Figure 2 is a scanning electron microphotograph (SEM) of corn-starch granules, several of which have surface pores. Figure 3 is an SEM illustrating the extreme pitting that results from α-amylase attack on corn-starch granules. Such pitting has not been observed with granules of CD-derived amyloses. Figures 4, 5 and 6 are SEMs of typical uncooked CD-derived granules. Their shapes are globular and their surfaces are rough, apparently the result of a binding

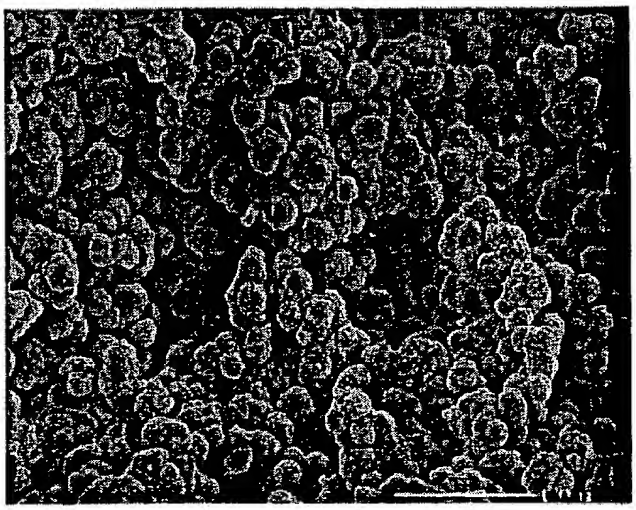

Figure 7 SEM of uncooked CD-derived amylose granules (prepared at 50 °C) that have been treated in aqueous solution with salivary α-amylase at 37 °C (× 1250)

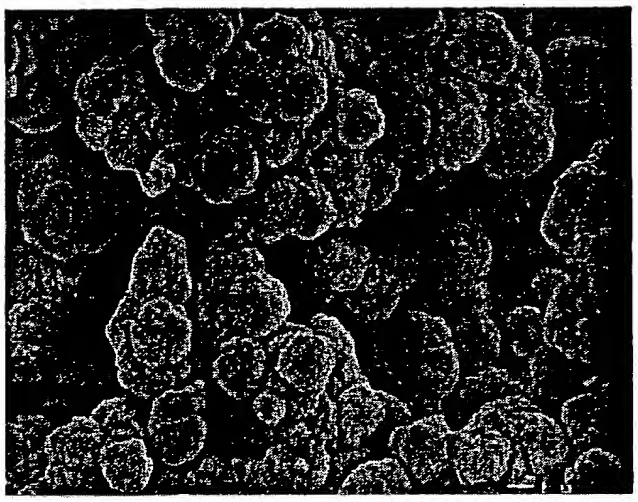

Figure 8 SEM of uncooked CD-derived amylose granules (prepared at 50 °C) that have been treated in aqueous solution with salivary α-amylase at 37 °C (× 2500)

together of numerous very small agglomerates to form much larger agglomerates, or granules. Figures 7 and 8 are SEMs of uncooked CD-derived amylose (prepared at 50 °C) that has been treated with α-amylase. The enzyme caused little change in the spherical character of the granules.

Granules of CD-derived amyloses prepared at low temperature (2–25 °C) were, in general, totally birefringent under a polarizing microscope. However, birefringence occurred only sporadically among granules prepared at 50–70 °C, which would suggest that high preparative temperatures might be detrimental to orderly arrangements of starch molecules in granular structures. It is not known at this time whether any relationship exists between granular birefringence and susceptibility of granules to enzymic hydrolysis.

Hydrolysis of cooked starches

Table 3 presents results of reactions of salivary α-amylase at 37 °C with starches that have been cooked in aqueous media for 30 min at 100 °C. During cooking, granular starches from ordinary corn, waxy maize or potato become swollen and undergo extensive disruption with concommitant liberation of starch molecules. This process, commonly called gelatinization, greatly lowers the resistance of starch to enzymic attack. Even when cooking merely causes swelling, rather than disruption, of starch granules, as with hybrid corn starches of relatively high amylose content (e.g. Hylon VII and Amylomaize VII), there could be significant diffusion of starch molecules from the swollen granules into the surrounding medium. The magnitude of this diffusion might be large enough to have caused the moderately high levels of enzymic interaction reported in Table 3 (reactions 4, 5, 6 and 7), although not so high as the levels observed for cooked starches from ordinary corn, waxy maize and potato (Table 3; reactions 1, 2, 3, 10 and 11). Consideration should also be given to the possibility that cooking alters the structure of granules from Hylon VII and Amylomaize VII and thereby promotes their penetration by α-amylase.

Cooked 100% corn amylose (Table 3; reaction 8) was hydrolysed by α-amylase to approximately the same extent (34.7%) in 8 h as was the uncooked form (35.3%) reported in Table 2. Increasing both reaction time and enzyme concentration had almost no effect on extent of hydrolysis (Table 3; reaction 9). Cooking apparently had little effect, if any, on the structure of the starch.

Low-$\bar{d}p$, CD-derived amyloses did not gelatinize when cooked in water at 100 °C. Dissolution in water at that temperature was only slight, being greatest with amylose prepared at 2 °C. Most of what dissolved at 100 °C precipitated in granular form very slowly over a period of hours when the mixtures were allowed to stand at room temperature. Reactions with α-amylase at 37 °C were initiated immediately after cooking operations to minimize the lessening effect that precipitation of amylose could have on the extent of hydrolysis. All of the cooked CD-derived amyloses (Table 3, see reactions 12–17) were hydrolysed much less effectively than cooked granules of natural starches (Table 3, see reactions 1–7, 10 and 11). Those amyloses prepared at 50 and 70 °C were the most resistant, averaging 22.6% degradation after an 8-h reaction in which the α-amylase activity was 13 units/ml of reaction mixture. In comparison, cooked starches of ordinary corn, waxy corn, Hylon VII, Amylomaize VII and potato underwent 81.8,

Table 3 Reaction of salivary α-amylase at 37 °C and pH 7.0 with cooked starches

Cooking was conducted at 100 °C for 30 min. Conditions: volume of mixture, 1 ml; starch, 40 mg (anhydrous weight; equivalent to 0.2467 mmol of glucose residues); Tris/HCl buffer, 0.01 M; $CaCl_2$, 1 mM. Abbreviations: G_1, D-glucose; G_2–G_4, straight-chain malto-oligosaccharides of dp 2–4; G_5–G_7, formed only in reactions where the substrate contained an appreciable amount of amylopectin, were probably *branched* glucans of dp 5, 6 and 7, respectively. Their HPLC elution times were significantly greater than the corresponding elution times of maltopentaose, maltohexaose and maltoheptaose. In reaction 1, a component of molecular mass greater than that of G_7, probably G_8 (dp 8), was indicated by HPLC analysis. Its contribution to overall yield of saccharides was estimated to be 2.8 mmol of glucose residues, which would raise the level of starch degradation to 77.8%.

Reaction no./starch	α-Amylase (units)	Time (h)	Saccharide yields (mmol of G_1 residues × 10^3)								Degradation of starch to G_1–G_7 (%)
			G_1	G_2	G_3	G_4	G_5	G_6	G_7	Combined	
Natural starches											
1. Corn, ordinary (≈ 25% amylose)	13	2	9.7	108.3	62.4	0	0.8	3.1	5.0	189.3	76.7
2. Corn, ordinary (≈ 25% amylose)	13	8	14.2	124.8	51.0	0	1.5	5.4	4.9	201.8	81.8
3. Waxy maize (99–100% amylopectin)	13	8	8.6	119.4	48.9	0	4.5	13.8	0	195.2	79.1
4. Hylon VII (hybrid corn; 64% amylose)	13	8	6.3	95.8	36.6	0	0	0	0	138.7	56.2
5. Hylon VII (hybrid corn; 64% amylose)	13	20	17.7	122.3	14.7	0	0.8	1.6	0	157.1	63.7
6. Hylon VII (hybrid corn; 64% amylose)	51	26	21.6	134.3	1.0	0	0	0	0	156.9	63.6
7. Amylomaize VII (hybrid corn; 66% amylose)	13	8	16.3	102.4	30.3	Trace	0.9	1.5	0	151.4	61.4
8. Amylose, 100% (non-granular; from corn)	13	8	6.1	60.6	18.9	0	0	0	0	85.6	34.7
9. Amylose, 100% (non-granular; from corn)	51	26	13.8	78.0	0.9	0	0	0	0	92.7	37.6
10. Potato	13	8	10.8	141.6	50.7	0	2.2	7.8	5.5	218.6	88.6
11. Potato	13	20	17.9	157.2	25.7	0	2.0	7.0	4.7	214.5	86.9
Amyloses, CD-derived, low-$\bar{d}p$											
12. 2 °C product	13	8	4.8	49.0	13.8	0.6	0	0	0	68.2	27.6
13. 25 °C product	13	8	5.1	64.2	21.0	0	0	0	0	90.3	36.6
14. 40 °C product	13	8	7.2	57.8	14.4	0	0	0	0	79.4	32.2
15. 50 °C product	13	8	4.7	39.6	8.1	0.7	0	0	0	53.1	21.5
16. 50 °C product	13	20	7.3	36.2	1.8	Trace?	0	0	0	46.1	18.7
17. 70 °C product	13	8	4.5	44.4	9.6	0	0	0	0	58.5	23.7

79.1, 56.2, 61.4 and 88.6% degradation, respectively, under the same conditions. Among the CD-derived amyloses, observed variations in resistance to α-amylase were possibly related, at least partly, to differences in granule solubility. Those amyloses with the higher solubilities would be expected to exhibit the greater susceptibilities to hydrolysis. Studies now in progress indicate an inverse relationship between average chain length ($\bar{d}p$) and solubility at 100 °C. This relationship might explain the higher resistance observed for amyloses prepared at 50–70 °C. Figure 9 is an SEM of a CD-derived amylose (prepared at 70 °C) that was cooked and subsequently treated with salivary α-amylase. The treatment had no observable effect on granular shape and very little effect on granular size.

The levels of hydrolysis reported in Table 3 for cooked CD-derived amyloses represent maximum levels. Treatment of the cooked amyloses with α-amylase at 37 °C was made before any significant retrogradation of dissolved amylose could occur subsequent to the temperature reduction from 100 to 37 °C. Any retrograded amylose would probably exhibit an amylolytic susceptibility as low as that of uncooked amylose. Granules of CD-derived amylose are possibly very similar in composition and physicochemical properties to so-called 'resistant starch', produced when ordinary starch gels or amylose gels are given prolonged treatment with α-amylase or mixtures of α-amylase and pullulanase. 'Resistant starch', prepared in this manner, is in the form of spherulites composed of highly organized linear glucans that possess a B-type X-ray diffraction pattern, a low solubility in water, and, frequently, a low $\bar{d}p$ (≈ 20–60) [16,19].

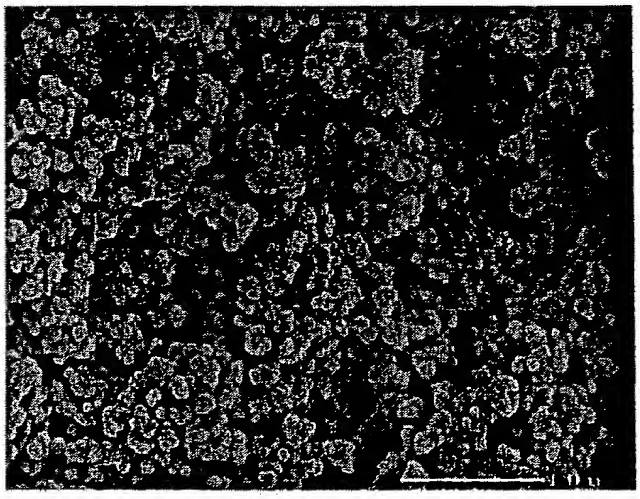

Figure 9 SEM of cooked CD-derived amylose granules (prepared at 70 °C) that have been treated in aqueous solution with salivary α-amylase at 37 °C (× 1250)

Acknowledgments

I am indebted to C. A. Knutson, Jr. for spectrophotometric determinations of amylose content and $\bar{d}p$ of starches; to F. L. Baker for scanning electron microphotography; and to G. D. Grose for X-ray diffraction analyses. Product names are necessary to report factually on available data; however, the U.S. Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. All programs and services of the USDA are offered on a non-discriminatory basis without regard to race, colour, national origin, religion, sex, age, marital status or handicap.

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CHARLES G. FERRARI.

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STARCH GELS

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Starch gels impart to food materials and food mixtures characteristics peculiar to the kind of starch present. For example, baked flour mixtures owe their properties to hydrated, heated starch as well as to the other constituents, and in cooked potato the starch determines very largely the texture and quality found there. Starch of some kind or another "thickens" the sauces, puddings and fillings which we eat daily; the choice of a starch for thickening purposes depends upon the flavor which the different starches possess and also upon the physical character of the paste or gel produced. The starches encountered most often are those of cereals and of roots, of which six kinds furnished material for observation in this study.

The character of the gel that a starch will yield and the conditions under which the gel forms, serve as means of observing the behavior of different starches. There are no satisfactory methods of measuring the stiffness of starch gels. Viscosity measurements can be applied to thin pastes but not to heavy ones containing 4% or more of starch, and plastometric means have not yet been developed for use with material of this structure. Photographs have served in this study to give permanent records of differences in physical appearance which could otherwise be described but inadequately. The pastes photographed contained 5% starch, in which concentration many kinds of starches will form gels capable of retaining the shape of a mold and therefore of being photographed. The outline of the gel indicated much of its character, though differences were noted in degree of opaqueness, tenderness and freedom from gumminess. Amounts of sucrose equal to those sometimes used in sweetening starch puddings were added to pastes, and the diminution of gel strength thereby produced was noted.

The records obtained in our study, of temperatures to which

pastes had to be heated before gelation was possible, contribute something to the present information on gelatinization temperatures of starches. The subject of gelatinization has occupied many investigators, the details of whose measurements have already been reviewed several times. (Reichert, 1913; Alsberg and Rask, 1924.)

Methods used have consisted of noting microscopic changes in the appearance of the granules, of observing temperatures at which translucency is altered, and of studying changes in viscosity of the paste. Gelatinization temperature has, in this study, been noted in connection with the ability of the cooled paste to form a gel. Our results are in agreement with the statement by Alsberg (1926) that the gelatinization change is continuous over a range of temperature. We have found maximum gelation to occur only after the paste has been heated to a temperature of 90° or above.

The syneresis of gels of varying starch concentration has been measured by Chapman and Buchanan (1930) in corn, wheat, rice and potato starch gels. They state that neither the rate nor the length of the period of heating the suspension affects rigidity or the amount of syneresis in the resulting gels. Hence all their pastes were heated quickly to boiling over a flame and poured out at once. It is not possible to say whether our gels, prepared somewhat differently, were similar in properties to the ones with which Chapman and Buchanan worked, because they confine their measurements to the amount of syneresis and offer no description of the gels' appearance.

Method of Study

Because starch thickening agents are used in the household in only fairly pure form, no attempt was made to purify the samples for these experiments. The cornstarch was a well-known brand of culinary type and the wheat, rice, potato, arrowroot and cassava starches were purchased through a laboratory supply house. In order that results might be interpreted correctly, analyses were made for moisture, ash and nitrogen, the results of which will be found in Table I. No significant amount of nitrogen was found in

TABLE I

ANALYSIS OF THE STARCHES USED

	Moisture %	Ash %	Nitrogen %
Corn	11.30	0.20	0.032
Wheat	10.25	0.13	0.026
Rice	10.80	0.70	0.071
Potato	14.29	0.35	0.049
Arrowroot	13.59	0.28	0.029
Cassava	10.63	0.15	0.012

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Nitrogen %
0.032
0.026
0.071
0.049
0.029
0.012

any starch; the moisture was about what would be expected in products marketed in paper packages. Ash content indicated that the starches were not pure, but they were so nearly so that differences in gel characteristics could be attributed to the starch component of the only fairly pure material. These observations will be repeated and extended later with purified instead of commercial starches.

One hundred grams of a 5% by weight suspension of starch in water was heated in a 150 cc. test tube which was immersed to a depth below the level of the suspension in a mechanically stirred water bath. The bath consisted of distilled water in a beaker behind which a strong light was placed. The light shining through it made changes in appearance of the contents of the test tube easily discernible. The deep, slender column of starch suspension insured uniform penetration of heat. By means of a glass rod bent to a paddle the starch was stirred occasionally by hand until the stage had been passed where granules were apt to settle to the bottom. Thermometers in the bath and in the tube indicated that there was a uniform difference in temperature between the two of 5° C. throughout the heating period until the bath reached 100°, after which 22 min. more were required to bring the temperature of the starch paste to 99.5° C. the maximum obtainable inside the test tube. At this rate of heating, the starch paste reached a temperature of 90° in about 38 min. The time and temperature intervals are given in the graph (Figure 1).

The tube of starch was removed from the bath after the desired interval of heating and the slight loss by evaporation was adjusted by the addition of water. The paste was viscous but fluid while hot; it was poured into small porcelain crucibles holding 10 g. of paste. Or, in some cases, only a part of the contents of the test tube were poured out and the heating of the remainder was continued for a time after which molds were filled again. At the end of 24 hours the gels were turned out of the molds onto watch crystals and examined for general appearance and firmness. One intact mold of each lot was reserved for photographing. The gels formed in less time than 24 hours but it was convenient to examine all samples one day after they were made.

In certain series sucrose was added to the cold starch suspension and the whole heated together. When 10, 30 and 60 g. of sucrose were added to the 100 g. of starch paste, the corresponding samples were said to contain 10, 30 and 60% of sucrose. It was desired that the ratio of starch to water be kept constant whether or not sucrose was present.

Discussion

Heating temperature required for gelation.—In the course of the gradual heating of the tube of starch suspension there occurred a continuous but slight diminution in opacity. There was visible, however, a quite sudden increase in translucency at a temperature which was specific for each starch. The temperature at which this change occurred was not a sharp one but ranged over 1 or 2 de-

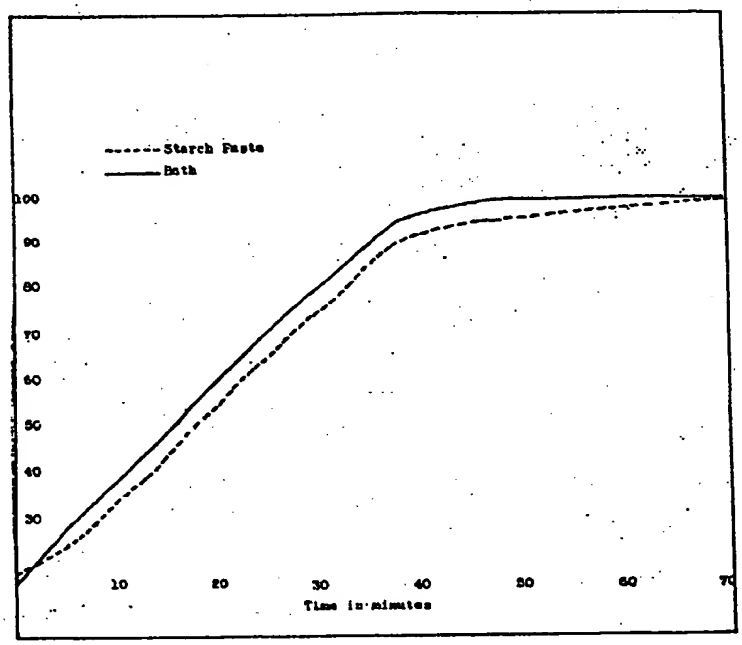

Fig. 1. Rate of Rise in Temperature of Starch Paste and Bath

grees. The paste seemed to have reached its maximum translucency at this temperature; heating either for a longer time or to a higher temperature made but slight difference in its color. This temperature corresponds to what some have called "gelatinization temperature" and for each kind of starch in 5% concentration [1] was:

Corn	86 to 87°C.	Potato	69 to 70°C.
Wheat	87 to 88°	Arrowroot	79 to 80°
Rice	84 to 85°	Cassava	74 to 75°

The change in translucency at what is called here the gelatinization temperature was accompanied by decided swelling of the

[1] It is likely that the temperature of gelatinization as evidenced by changes in translucency varies with the concentration of starch.

starch granule as shown by microscopic examination, and there was also apparently a change in viscosity under these conditions, though no measurements on the latter are being reported at this time. This temperature was insufficient, however, to permit maximum swelling of the granules, without which seemingly a gel will not form. Samples were poured into molds at the temperatures given above for each starch; the cooled pastes showed evidence of having thickened somewhat at this temperature (bottom row, Plate I) but the gel was weak, granular and showed much syneresis. No evidence of gelation appeared in pastes which had been heated to temperatures 1° or 2° below those given.

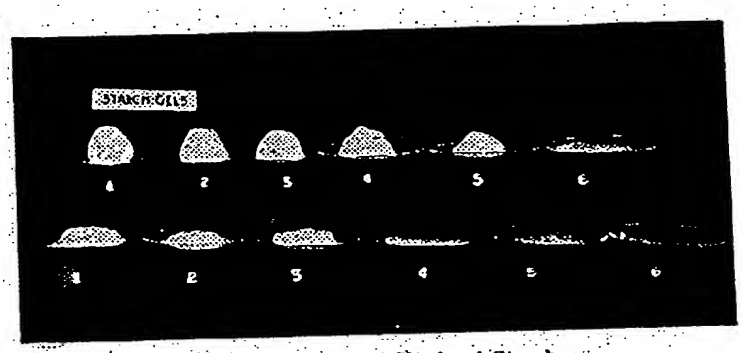

Plate I. Gels of Six Kinds of Starch

1. Corn; 2. Wheat; 3. Rice; 4. Potato; 5. Arrowroot; 6. Cassava.
Above: All pastes had been heated to 99.5° C.
Below: Pastes had been heated to 87°, 88°, 85°, 70°, 80° and 75°, respectively.
(Visible changes in translucency of the pastes occurred at these temperatures.)

Continuing the heating to 99.5° C. gave the three very well-formed cereal starch gels of the top row in Plate I and the soft or quite fluid gels of the root starches also pictured in the top row. Of the three cereal starch gels, the rice was most translucent and most tender as it was cut; cornstarch gave the firmest gel and was chalky white in color even though its hot paste had been a translucent blue. Wheat starch gave results intermediate in value. The potato starch paste was ropy and almost water-clear; the cold gel was quite transparent, too gummy to leave the mold well and in all a poorly formed gel. Arrowroot gave a still more transparent and softer gel. The cassava starch paste was only a viscous fluid even when heated to 99.5°.

If the maximum strength of a gel is desired in a starch, its paste must be heated beyond the temperature at which its translucency suddenly increases. This result suggested that perhaps long continued heating at the gelatinization temperature might

bring about maximum gelation. Each starch was therefore heated in turn for 15 and 30 minutes at its gelatinization temperature; this improved gelation somewhat but did not yield a gel which even approached in firmness the one which had been heated to 90° and removed immediately. Heating the paste to 90°, 95° and 99.5° gave gels indistinguishably alike. This manner of finding the conditions for maximum gel strength was used with each starch in turn with the same general result. Only the wheat starch is so represented in the plates. (Plate II.)

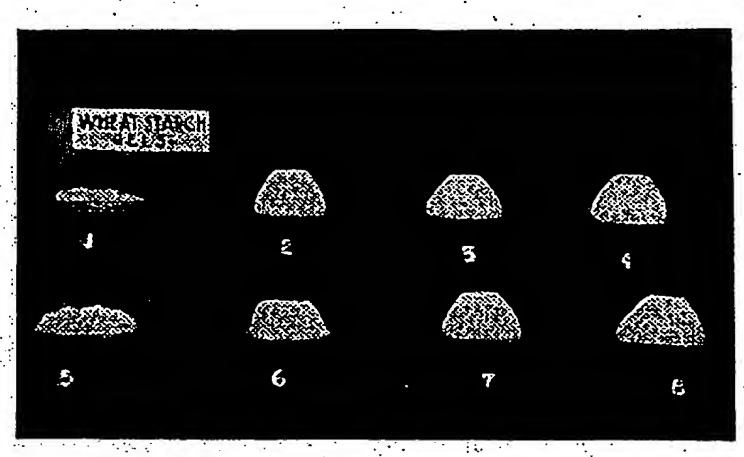

Plate II. Effect upon Gel Formation of Time and Temperature of Heating

1. Paste was heated to 88° C.
2. Paste was heated to 90° C.
3. Paste was heated to 95° C.
4. Paste was heated to 99.5° C.
5. Heating continued at 88° C. for 15 min.
6. Heating continued at 88° C. for 30 min.
7. Heating continued at 99.5°C. for 15 min.
8. Heating continued at 99.5° C. for 30 min.

Most starch mixtures probably reach a temperature of 95° to 100° in the course of ordinary cooking. Forty or more records on the inner temperature of baked potatoes have given 100° to 104° as the one for "doneness"; at an inner temperature of 97° the potato seemed still underdone. Biscuits, muffins and butter cake reached a temperature of 100°, yeast bread 98° and even angel food cake, containing as it does large amounts of easily over-coagulable egg white, has been found to have attained an inner temperature of 97°, 98°, 99° and 100° in four different bakings.[2] Other factors than the gelation of the starch of course contribute to "doneness" in the above products but there is little likelihood that incomplete gel formation occurs commonly, since it has just been shown that a temperature lying between 87° and 90° is sufficient for complete

[2] Unpublished results by Woodruff.

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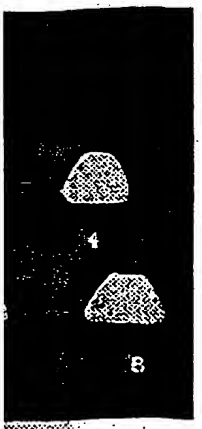

Temperature of Heating
d at 88° C. for 15 min.
d at 88° C. for 30 min.
d at 99.5° C. for 15 min.
d at 99.5° C. for 30 min.

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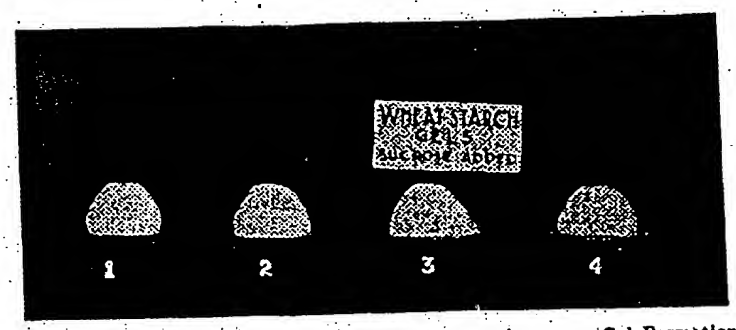

Plate III. Effect of Increasing Sucrose Concentration upon Gel Formation

1. Sucrose none
2. Sucrose 10 percent
3. Sucrose 30 percent
4. Sucrose 50 percent

Plate IV. Effect of Sucrose upon Gels of Six Starches

1. Corn; 2. Wheat; 3. Rice; 4. Potato; 5. Arrowroot; 6. Cassava
Above: Gels containing 30 per cent sucrose, heated to 99.5° C.
Below: Gels containing 60 per cent sucrose, heated to 99.5° C.

Plate V. Effect of Reducing the Starch Concentration per Unit of Volume by Adding Sucrose and by Adding Water

Left: Contains 5 grams of wheat starch and 95 grams of water.
Center: Contains 5 grams of wheat starch and 182 grams of water.
Right: Contains 5 grams of wheat starch, 95 grams of water and 60 grams of sucrose.
The volumes of center and right were the same.

gelation of wheat and corn starches. In rice the temperature lies between 85° and 90° and in potato between 70° and 80°. Arrowroot and cassava formed very poor gels which were little improved by heating to a higher temperature.

Effect of sucrose upon gels.—The failure of starch-thickened fillings to gel in the presence of large amounts of sugar is not infrequently noted. In studying the conditions under which such an effect is produced, additions of different quantities of sucrose were made to pastes, all of which were heated to 99.5° and the resulting gels photographed. Gels containing successively 10, 30 and 50% sucrose showed increasing transparency and tenderness but were well-formed in the three cereal starches. When 60% sugar was added, however, a viscous, syrupy mass resulted but no gel. Plate III, illustrating wheat starch, shows that there is very little outward effect upon the starch of amounts of sucrose up to 50%. Plate IV pictures the rigidity of gel structures of the six starches in the presence of each 30 and 60% sucrose. The three root starches had formed very weak gels even in the absence of sugar; when so little as 10% sucrose was added to their pastes a noticeable softening occurred and 30% flattened out the gel considerably. A syrup was the result when 60% sugar was used with the root starches, as was also the case in the cereal starch pastes.

The volume of a starch-sugar paste containing 5 g. of starch, 95 g. of water and 60 g. of sucrose was 132 cc., whereas the volume of the same amounts of starch and water was approximately 100 cc. The increase in volume had reduced the starch from 5 to 3.8% on the basis of volume. Such a reduction in the ratio of starch to volume would account for some diminution in gel strength but not for the progressive change in color and translucency which was noted as more and more sugar was added. There is evidence in the series of wheat starch gels in Plate V that the sucrose did have a specific effect upon gelation. Photographed there is the syrupy fluid of the paste containing 60% sucrose and a tender but well-formed gel obtained when a starch-water paste was diluted with water to 132 cc., the volume of the sugar-containing one. For purposes of comparison, a gel containing 5 g. of wheat starch and 95 g. of water is also shown in Plate V.

The mechanism of this action by sucrose can only be speculated upon in the light of the present findings. It is possible that the presence of large amounts of sucrose prevents the starch granules from imbibing the water needed for their swelling. The amounts of sucrose employed here are none of them beyond the limits of

what is used in cooked starch mixtures. The cause of the behavior of sucrose is being studied now by other means.

Summary

When starch-water pastes containing 5% of either corn, wheat, rice, potato, arrowroot or cassava starches were heated only to the temperatures at which the different ones exhibited marked changes in translucency, the cooled pastes did not form gels strong enough to retain the shape of a mold. Maximum gel strength was obtained in each starch at a temperature of 90° or higher. The cereal starch gels were well-formed and had clearly defined outline; root starches gave poorly-formed gels. Photographs constituted a permanent record of the appearance of these gels.

Maximum gelation of starch very probably occurs in baked flour mixtures, in cooked potatoes and in flour-thickened sauces. The final inner temperature of such products has in many cases been observed to be at or near 100° C.

The presence of sucrose in amounts as great as 60% of the weight of the starch paste reduced the mass to a viscous syrup. A starch-water paste of the same volume did not behave as did the starch-sugar-water one. This effect of sucrose is being studied further.

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;sistant starch: the effect on postprandial glycemia, rmonal response, and satiety[1–3]

e Raben, Anna Tagliabue, Niels J Christensen, Joop Madsen, Jens J Holst, and Arne Astrup

TRACT The effect of resistant starch (RS) on postpran- plasma concentrations of glucose, lipids, and hormones, and ıbjective satiety and palatability ratings was investigated in ealthy, normal-weight, young males. The test meals con- d of 50 g pregelatinized starch (0% RS) (S) or 50 g raw to starch (54% RS) (R) together with 500 g artificially sweet- syrup. After the R meal postprandial plasma concentrations ucose, lactate, insulin, gastric inhibitory polypeptide (GIP), agon-like peptide-1, and epinephrine were significantly :r compared with after the S meal. Moreover, subjective :s for satiety and fullness were significantly lower after the eal than after the S meal. Differences in GIP, texture, and lability may have been involved in these findings. In conclu- , the replacement of digestible starch with RS resulted in ificant reductions in postprandial glycemia and insulinemia, in the subjective sensations of satiety. *Am J Clin Nutr* 1;60:544–51.

Y WORDS Potato starch, appetite, palatability, lactate, lin, gastric inhibitory polypeptide, catecholamines

oduction

ntil recently, starch was believed to be 100% digested in the ll intestine—independent of the source, type, and preparation ıe starch. However, within the past 10 y it has been found . despite the fact that pancreatic α-amylase is present in the in ample amounts (1), a fraction of the ingested starch passes igested to the large bowel (2–4). Here the starch undergoes ore or less complete fermentation, resulting in the production uptake of short-chain fatty acids (acetic acid, butyric acid, propionic acid) (5, 6). This fraction of the starch has been ed resistant starch (RS) and has been defined by the Euro- ı FLAIR Concerted Action on Resistant Starch (EURESTA) 'the sum of starch and products of starch hydrolysis not ab- ed in the small intestine of healthy individuals."

he amount of RS present in starch-rich foods depends on eral factors, ie, the source, ripeness, processing, preparation, storage of the foods. It has been shown that starch from white id, porridge oats, and cornflakes is almost completely di- ed in the small intestine (4) whereas native starch from ba- a and uncooked potato is highly resistant to hydrolysis in vitro and in vivo (8). When the potato is cooked the starch granules ıtinize and become readily digestible, whereas cooling of the ıto reverses this gelatinization process and renders ≈12% of starch resistant to small intestinal digestion (9).

The importance of RS to human health, ie, diabetes, overweight, cardiovascular diseases, or cancer, is still not known. However, because RS—unlike nonresistant starch—is not digested and therefore not absorbed as glucose in the small intestine of healthy humans (8, 9), a reduction in both postprandial glycemia and insulinemia can be expected after the intake of RS compared with digestible starch. Such an effect of RS may be beneficial in the control of diabetes. Moreover, RS may through mechanisms similar to those exerted by soluble dietary fiber influence the amount and rate of absorption of other nutrients in the diet, ie, glucose and fat, which may be beneficial in the control of glycemia or lipidemia.

The potential use of RS as a weight-reducing agent may also be of interest because the energy value of 1 g RS, including the contribution from fermentation products, has been estimated to be only 9.0–9.8 kJ/g, ie, half the value of digestible starch (10). It is therefore tempting to suggest RS as a weight-loss agent; however, the impact of RS on macronutrient balance and appetite control may be less beneficial to weight regulation. This hypothesis is based on the recently developed concepts on the regulation of energy and macronutrient balance. Thus, there seems to be a close regulation between the body's macronutrient stores and the three macronutrients (fat, protein, and carbohydrate) in the diet (11, 12). The maintenance of the body's relatively small carbohydrate stores seems especially crucial to overall energy balance (11). Moreover, recent studies have shown that the control of appetite may be influenced by the amount of available carbohydrate in the diet (13, 14) and that consumption of a certain amount of carbohydrate (or protein) in a meal may be necessary to achieve satiation (15). Increasing the amount of RS in the diet with a resultant decreased amount of absorbed carbohydrate and a decreased carbohydrate-to-fat ratio in the diet may thus lead to

[1] From the Research Department of Human Nutrition, Centre for Food Research, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark; the Department of Human Nutrition, University of Pavia, Italy; the Department of Internal Medicine and Endocrinology, Herlev Hospital, Denmark; and the Department of Physiology, The Panum Institute, University of Copenhagen, Denmark.

[2] Supported by grants from The Danish Research and Development Programme for Food Technology 1990–1994 and the Danish Medical Research Council grant 12-9537-3.

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TABLE 1
Subject characteristics[1]

	Denmark (n = 5)	Italy (n = 5)	All (n = 10)
Age (y)	25.6 ± 1.9	21.2 ± 0.7	23.4 ± 1.2
Height (m)	1.80 ± 0.01	1.78 ± 0.02	1.79 ± 0.01
Weight (kg)	71.2 ± 3.0	74.0 ± 3.5	72.6 ± 2.2
BMI[2]	22.0 ± 1.1	23.2 ± 0.6	22.6 ± 0.6
Fat mass (%)	18.6 ± 2.4	20.8 ± 1.3	19.7 ± 1.3

[1] $\bar{x}$ ± SEM.
[2] In kg/m^2.

a reduction in the satiating power of the diet. This may result in overconsumption and subsequent weight gain. However, if RS has a "dietary-fiber-like" positive effect on satiety (16–18) an increase in the amount of RS in the diet may be beneficial to weight maintenance.

The purpose of the present study was to investigate the acute effects of RS vs digestible nonresistant starch on postprandial changes in glycemia, hormonal response, and subjective sensations of hunger and satiety. The two test starches were given in as pure a form as possible to clarify the role of the starches per se without the interference from other nutrients.

Subjects and methods

The experiment was performed as a joint study within the EURESTA framework. Exactly the same experiment was conducted at two centers: the Research Department of Human Nutrition, The Royal Veterinary and Agricultural University, Denmark, and the Department of Human Nutrition, University of Pavia, Italy.

Subjects

In each center five healthy male subjects (20–31 y of age, normal-weight, nonsmokers, not elite athletes) with no history of obesity or diabetes, participated in the study (Table 1). Females were not included to avoid possible differences due to the menstrual cycle. The subjects' energy needs during the study were determined by using WHO tables according to age, weight, height, and sex. The multiplication factor 1.78 was used to account for medium physical activity level of the subjects (19). The body composition of the subjects was estimated by the bioimpedance method by an Animater (HTS Engineering Inc, Odense) in Denmark and BIA 109 (RJ1 Systems, Detroit) in Italy. Fat-free mass was calculated by using the equation of Deurenberg et al (20). The study was approved by the Municipal Ethical Committee of Copenhagen and Frederiksberg to be in accordance with the Helsinki-II declaration and all subjects gave written consent after the experimental procedure had been explained to them.

Diets

The two test meals consisted of 50 g raw potato starch (54.1% RS) (R) or 50 g pregelatinized potato starch (100% digestible) (S) mixed into 500 mL diluted artificially sweetened fruit syrup (Table 2). The syrup was based on apple, grape, red current, elderberry, black current, and cherry and commercially purchased from Irma, Denmark. The starches were produced and supplied from l'Institut National de la Recherche Agronomique (INRA), Nantes, France. Both starches appeared as a white flour. Just before consumption the starch was slowly poured into the syrup while mixing it with a hand mixer at the slowest speed to avoid an increase in temperature of the mixture. Several other ways of administration were tested before deciding on these test meals. However, the pregelatinized starch could not be mixed into other liquids without producing a very unpleasant, inedible substance. Because heating would affect the physical properties of the raw starch, a cold-meal preparation had to be used. The starches could thus not be baked into a bread or used in a pasta. The best compromise for these two starches was therefore to mix them with the syrup. For the R meal this resulted in a drinkable mixture whereas the S meal was a porridge-like gel to be eaten by spoon. Although the syrup was sweetened with artificial sweeteners it contained some fruit sugars naturally occurring in the berries and fruits (Table 3).

Each test day was preceded by 3 d on an identical carbohydrate-rich diet (60% of energy as carbohydrate, 28% as fat, and 12% as protein, and 3.5 g dietary fiber/MJ), prepared at the departments from food items according to each subjects' individual energy requirements, and adjusted to the nearest 0.5 MJ. The subjects were instructed to adhere strictly to the diet. If subjects

TABLE 2
EURESTA reference starch materials[1]

Food	DM	Calculated starch values TS	RDS	SDS	RS
	%	% by wt			
Raw potato starch	83.4	81.3	6.0	21.2	54.1
Pregelatinized potato starch	95.1	92.9	87.9	5.2	—

[1] DM, dry matter; TS, total starch; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch. Analyzed from the Dunn Clinical Nutrition Centre. Methods described by Englyst et al (21) and Englyst and Cummings (22).

TABLE 3
Carbohydrate and energy contents in the two test meals (50 g potato starch + 500 mL diluted fruit syrup)[1]

	Test meal R	S
Total starch in 50 g potato starch (g)	40.7	46.5
RS (g)	27.1	0.0
RDS (g)	3.0	44.0
SDS (g)	10.6	2.6
Fruit sugars from syrup (g)	8.4	8.4
Glucose (g)	2.9	2.9
Fructose (g)	4.4	4.4
Sucrose (g)	1.1	1.1
Total digestible carbohydrate (g)[2]	22.0	54.9
Total energy (kJ)[3]	367	917

[1] R, raw potato starch; S, pregelatinized potato starch; RS, resistant starch; RDS, readily digestible starch; SDS, slowly digestible starch.
[2] Not taking fermentation into consideration.
[3] 16.7 kJ/g.

could not consume all the food, they had to bring the leftovers to the departments for weighing and registration. The same food was deducted from the diet during the following preexperimental periods. The subjects were instructed to abstain from strenuous physical activity for the 2 d before the test days. Together with the standard diet this should ensure equally filled glycogen stores and similar macronutrient balance on the 2 test days. The computer databases of foods from the National Food Agency of Denmark (Dankost) and Italy (INN) were used in the calculations of energy and nutrient composition of the test diets.

Experimental protocol

The two test meals were given in a crossover design on separate days with ≥ 1 wk and no more than 6 wk separating the test days. On the test day the subjects arrived at the institute at 0800, with a minimum of physical activity, by car, bus, or train after having fasted for 12 h from the evening before. After subjects voided and were weighed (to the nearest 100 g), bioimpedance was measured. The subjects then rested in the supine position on a bed covered with an antidecubitus mattress with slight elevation of the head. A Venflon catheter (Viggo, Gothenborg, Sweden) was inserted in an antecubital arm vein. After a 10-min rest a fasting blood sample was taken and after a further 20-min rest resting metabolic rate was measured by indirect calorimetry by using a ventilated hood. A second fasting blood sample was taken hereafter and the test meal was then served and consumed within 10 min. Exactly the same time was spent on the two test meals for each individual subject. Postprandial energy expenditure was measured for 5 h (1000–1500 h), and blood samples were taken 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, and 300 min after the meal was consumed. During the postprandial measurements the subjects were allowed to watch light entertainment movies, and to have a break of a maximum of 5 min after 2 and 4 h. During the break the subjects could sit, walk quietly, or go to the toilet. The exact time schedule was noted and repeated on the following test days. Water consumption during the test period was allowed, but the total amount consumed was noted and repeated on the second test day.

Immediately before and every 30 min after the meal, questionnaires to assess hunger, satiety, fullness, and prospective consumption were filled out by each subject. Ratings were made on 100-mm visual analogue scales (VAS) with words anchored at each end, expressing the most positive (ie, good, pleasant) or the most negative ratings (ie, bad, unpleasant) (23). Immediately after the test meals the palatability, taste, aftertaste, texture, and visual appeal of the two test meals were recorded by the subjects using VAS scores. Data on energy expenditure are being published separately (A Tagliabue, A Raben, ML Heijnen, P Deusenberg, E Pasquali, A Astrup, unpublished observations, 1994).

Laboratory analyses

Blood was sampled without stasis through the indwelling antecubital cannula by using iced syringes. Plasma glucose and lactate were analyzed by standard enzymatic methods (24). Plasma glycerol was analyzed after trichloracetic acid precipitation, essentially as described by Chernick (25). Blood for determination of plasma catecholamines was collected in tubes containing ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and glutathione. Samples were immediately centrifuged for 10 min at 3000 × *g* and 4 °C and the plasma stored at −80 °C until determination of catecholamines by a radioenzymatic method (26). Immunoreactive insulin concentrations were measured in plasma with radioimmunoassay kits purchased from Novo, Copenhagen. Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) were determined by radioimmunoassay on plasma extracted with ethanol as previously described (27–29). Samples for the GIP and GLP-1 analyses could not be taken from the Italian subjects. The starch analyses were performed at the Dunn Clinical Nutrition Centre (United Kingdom) by using the methods described by Englyst et al (21) and Englyst and Cummings (22).

Statistical analyses

All results are given as means ± SEM. Data are presented as changes from basal fasting concentrations by using the second fasting blood sample as the basal concentration. Responses to the two test meals were compared by parametric analysis of variance (ANOVA) for repeated, paired measures with time and diet as factors and with subject nested into diet. The key results are presented with F or t values and degrees of freedom. Areas under the curves (AUCs) for the 5-h measurement periods were calculated separately for each subject as the difference between the integrated area of the response curve and the rectangular area determined by the basal values (= net response). A paired t test was used in the comparisons between two means on the same subject. Regression analyses were performed on the differences between the S and R meals (referred to as S−R) for the 5-h AUCs and means for the VAS scores, peak/nadir values, and Δpeak/Δnadir values. This was done to account for the data being paired. The level of significance was set at $P < 0.05$. *Statgraphics* software version 4.2 (Graphic Software Systems, Inc, Rockville, MD) and the *Statistical Analysis Package* (SAS Institute, Cary, NC) were used in the statistical calculations.

Results

Glucose and lactate

Fasting plasma glucose concentrations averaged 4.97 ± 0.08 mmol/L before the S meal and 4.91 ± 0.07 mmol/L before the R meal (NS). A significant interaction between diet and time was observed after the test meals with glucose concentrations increasing nine times as much after the S meal as after the R meal (Δpeak concentrations: S, 3.07 ± 0.29 mmol/L after 30 min; R, 0.36 ± 0.13 mmol/L after 15 min) ($F_{[12,216]} = 25.2$, $P < 0.0001$) (Fig 1). The 5-h AUCs averaged 79.53 ± 27.26 mmol·min/L after the S meal and −42.02 ± 9.51 mmol·min/L after the R meal ($t_9 = 4.51$, $P = 0.0015$). The AUCs for the first 2 h also resulted in a negative area for the R meal (−13.37 ± 6.71 mmol·min/L) compared with the S meal (140.00 ± 21.53 mmol·min/L) ($t_9 = 7.11$, $P < 0.0001$).

Plasma lactate concentrations increased after both test meals with a peak after 45 min for the S meal (1.36 ± 0.09 mmol/L), and after 15 min for the R meal (1.18 ± 0.10 mmol/L) (interaction diet-time: $F_{[12,216]} = 25.2$, $P < 0.0001$) (Fig 1). The increase from basal was twice as high after the S (0.52 ± 0.06 mmol/L) than after the R meal (0.24 ± 0.06 mmol/L). Five-hour AUCs averaged 6.9 ± 10.0 mmol·min/L after the S meal and −26.3 ± 8.9 mmol·min/L after the R meal ($t_9 = 3.70$, $P = 0.005$).

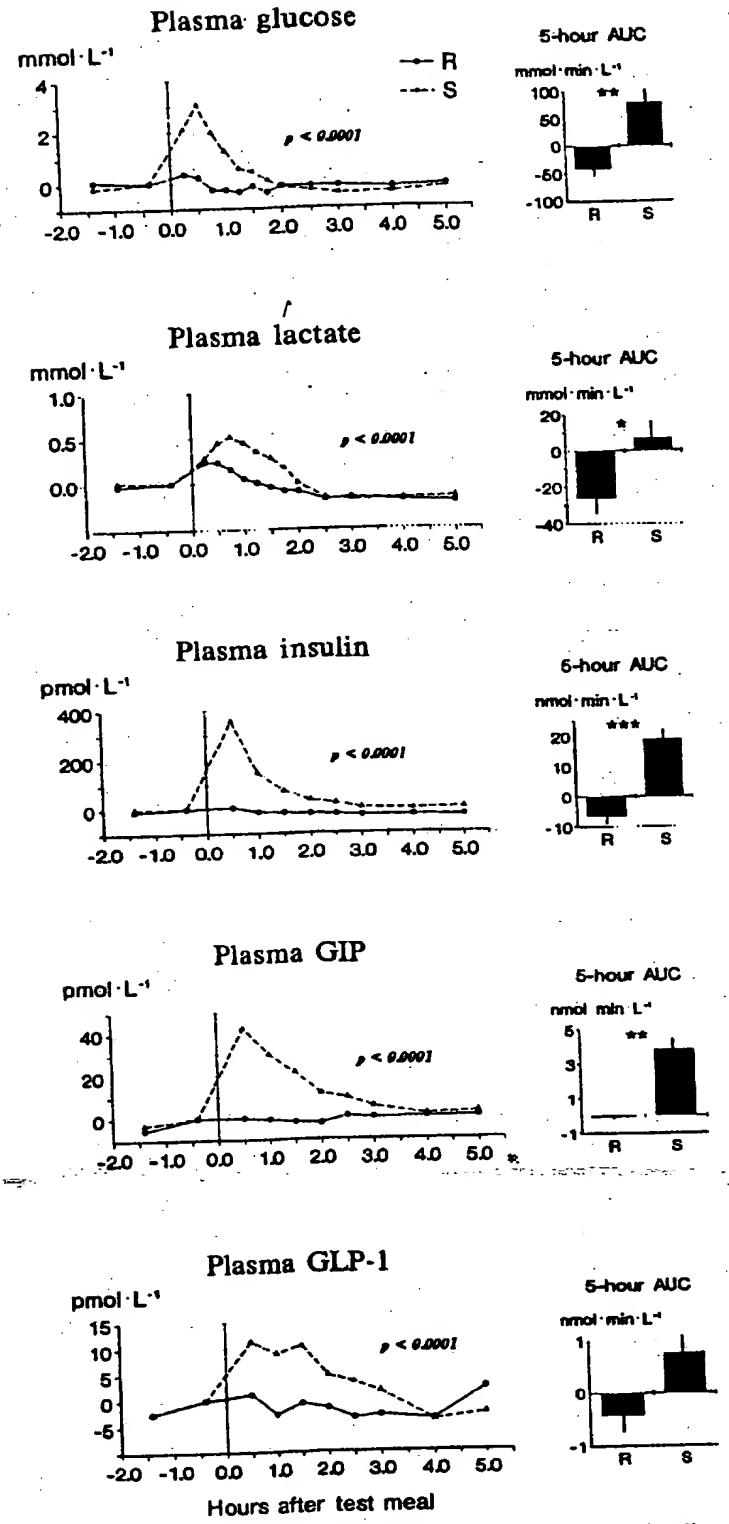

FIG 1. Change in plasma concentrations of glucose, lactate, insulin, gastric inhibitory polypeptide (GIP), and glucagon-like peptide-1 (GLP-1) after a raw potato starch meal [54% resistant starch (RS)] (R) and a pregelatinized potato starch meal (0% RS) (S) in 10 healthy, normal-weight, male subjects. All data are mean (±SEM), expressed as differences from fasting concentrations. Left panel (ANOVA): all (time effect: $P < 0.0001$); plasma glucose (diet effect: $F_{(1,18)} = 25.1$, $P < 0.0001$; interaction between diet and time: $F_{(12,216)} = 25.2$, $P < 0.0001$); plasma lactate (diet effect: $F_{(1,18)} = 36.7$, $P < 0.0001$; interaction between diet and time: $F_{(12,216)} = 25.2$, $P < 0.0001$); plasma insulin (diet effect: $F_{(1,18)} = 39.9$, $P < 0.0001$; interaction between diet and time: $F_{(8,143)} = 15.1$, $P < 0.0001$); plasma GIP ($n = 5$) (diet effect: $F_{(1,8)} = 27.3$, $P < 0.0001$; interaction between diet and time: $F_{(8,63)} = 14.6$, $P < 0.0001$); plasma GLP-1 ($n = 5$) (diet effect: $F_{(8,57)} = 4.7$, $P < 0.0001$). Right panel: areas under the curves (AUC). *** $P < 0.05$, ** $P < 0.01$, * $P < 0.001$.

Insulin, GIP, and GLP-1

Fasting plasma insulin concentrations averaged 53.7 ± 5.0 pmol/L before the S meal and 75.4 ± 10.0 pmol/L before the R meal (NS). A significantly different response pattern was observed after the two test meals. Compared with fasting concentrations plasma insulin increased by a factor of six to 355.4 ± 68.5 pmol/L after the S meal, but to only 84.6 ± 7.2 pmol/L after the R meal (interaction diet-time: $F_{(8,143)} = 15.14$, $P < 0.0001$) (Fig 1). The AUCs averaged 19.1 ± 4.0 nmol·min/L after the S meal and −6.6 ± 1.2 nmol·min/L after the R meal ($t_9 = 4.90$, $P = 0.0008$). Also, the AUCs for the first 2 h resulted in differences between the S (18.4 ± 2.8 nmol·min/L) and the R meals (−1.4 ± 1.1 nmol·min/L) ($t_9 = 5.34$, $P = 0.0005$).

Plasma GIP showed the same response pattern as plasma insulin (Fig 1). Thus compared with fasting values, GIP increased by a factor of 10 after the S meal whereas no increase was observed after the R meal (interaction diet-time: $F_{(8,63)} = 14.6$, $P < 0.0001$). The AUC S−R for GIP and lactate were positively correlated ($r = 0.91$, $P = 0.03$).

Plasma GLP-1 increased significantly after the S meal whereas no changes were observed after the R meal (interaction diet-time: $F_{(8,57)} = 4.7$, $P = 0.0002$) (Fig 1). The difference in the AUCs after the R and the S meals was nearly significant ($t_9 = 2.56$, $P = 0.06$). The AUC S−R for GLP-1 was negatively correlated with GIP ($r = -0.94$, $P = 0.02$) and lactate ($r = -0.88$, $P = 0.052$) and ΔpeakS−R for GLP-1 negatively correlated with GIP ($r = -0.91$, $P = 0.03$).

Triglycerides (TG) and glycerol

Plasma triglyceride concentrations showed a similar response pattern after the two test meals (Fig 2). Thus, TG decreased slightly after both meals with a nadir after 2.5 h and returned to baseline after 5 h (time effect, $F_{(9,135)} = 4.1$, $P < 0.001$). Triglyceride concentrations were significantly correlated with insulin for the S−R AUCs ($r = 0.65$, $P = 0.04$) and the S−R Δpeak/Δnadir values ($r = 0.83$, $P = 0.006$).

A tendency to a different response pattern for plasma glycerol was found after the two test meals. Thus plasma glycerol decreased after the S meal to a nadir after 1.5 h whereas no decrease was observed after the R meal (interaction diet-time: $F_{(8,144)} = 1.9$, $P = 0.06$) (Fig 2). After 5 h glycerol concentrations had increased by 59.3 mmol/L for the S meal and by 39.8 mmol/L for the R meal compared with fasting concentrations. The AUCs were not significantly different after the two test meals. The AUC S−R for glycerol was negatively correlated with glucose ($r = -0.75$, $P = 0.01$), whereas peak S−R for glycerol was positively correlated with peak S−R triglyceride ($r = 0.72$, $P = 0.02$).

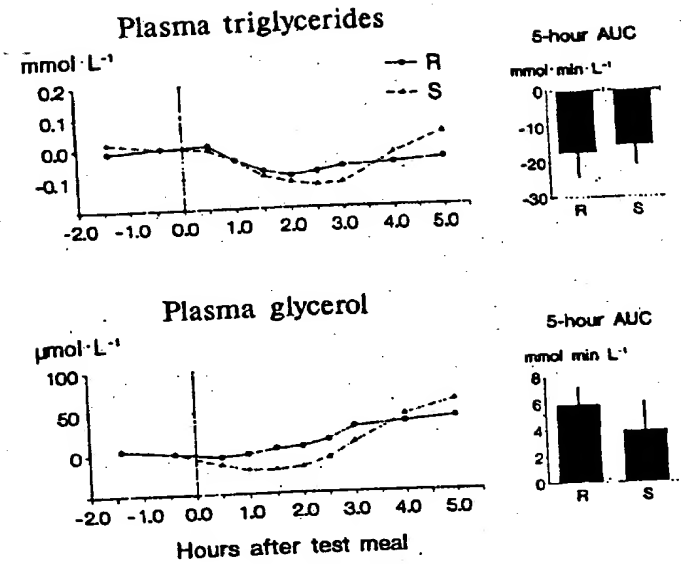

FIG 2. Change in plasma concentrations of triglycerides and glycerol after a raw potato starch meal (54% resistant starch [RS]) (R) and a pregelatinized potato starch meal (0% RS) (S) in 10 healthy, normal-weight, male subjects. All data are mean (±SEM) expressed as changes from fasting concentrations. Left panel (ANOVA): all (time effect: $P < 0.001$); plasma glycerol (interaction between diet and time: $F_{[9,144]} = 1.9$, $P = 0.064$). Right panel: areas under the curves (AUC).

Epinephrine and norepinephrine

No significant differences were observed in plasma norepinephrine (NE) after the two test meals (Fig 3). However, a significantly different response pattern for E was found after the two meals. Thus, no changes in plasma E concentrations were observed after the R meal, whereas E increased significantly from 2 to 4 h after the S meal (interaction diet-time: $F_{[9,144]} = 2.68$, $P = 0.0064$) (Fig 3). The differences in AUCs were not significantly different for E or NE.

Satiety scores

Significant differences were found for satiety and fullness (interaction diet-time: $F_{[11,198]} = 1.95$, $P = 0.03$ and $F_{[11,198]} = 2.30$, $P = 0.012$) after the two test meals (Fig 4). Thus the subjects felt more satisfied and more full after the S meal than after the R meal.

Simple regression analysis showed no correlations between any of the mean satiety scores and the blood indexes when 5-h AUCs were used. When peak and nadir values were included, mean hunger S−R was significantly correlated with Δpeak S−R for lactate ($r = 0.68$, $P = 0.03$) whereas peak GIP S−R was correlated with peak fullness ($r = -0.87$, $P = 0.057$) and with nadir prospective food consumption ($r = 0.88$, $P = 0.046$).

Evaluation of the test meal

The subjects found that the S meal looked less appetizing ($t_9 = 3.52$, $P < 0.001$), had a more unpleasant taste ($t_9 = 2.84$, $P < 0.05$), and had a firmer texture ($t_9 = -5.38$, $P = 0.0004$) than the R meal (Table 4). They did not find that the aftertaste or overall palatability were different between the meals. Simple regression analysis on the S−R scores showed correlations between aftertaste and mean satiety ($r = 0.65$, $P = 0.04$), hunger ($r = -0.64$, $P = 0.049$), and fullness ($r = 0.62$, $P = 0.054$).

Discussion

Marked differences in plasma concentrations of substrates and hormones as well as in palatability and satiety scores were observed after the two test meals. Overall, the S meal greatly stimulated plasma concentrations of glucose, insulin, and gastrointestinal hormones whereas no or only a modest stimulation of these indexes was observed after the R meal.

Plasma glucose increased nine times more after the S meal than after the R meal. This was not entirely unexpected on the basis of the analytical data on digestibility of the two test starches. However, the differences in postprandial glycemia were somewhat larger than would be expected from the clinical analyses. Thus, the ratio between the peak glucose response after the R and S meal was 1:9 (0.36/3.07 mmol/L), whereas the ratio between the amount of readily digestible carbohydrate was 1:5 (11.4/54.4 g) (Table 3). The reason for this difference is not readily apparent but may be connected with differences in the stimulation of gastrointestinal factors and insulin after the two meals.

Despite the minor increase in plasma glucose after the R meal, the increase in plasma lactate concentration amounted to 50% of the increase after the S meal. This may be due to the fructose present in the test meal (4.4 g) or to a part of the glucose being converted to lactate via nonoxidative pathways in the splanchnic region (30).

Also, plasma insulin increased markedly after the S compared with the R meal, producing a ratio between Δpeak values of 30:1 (182/6 pmol/L). This difference in insulin response is far greater

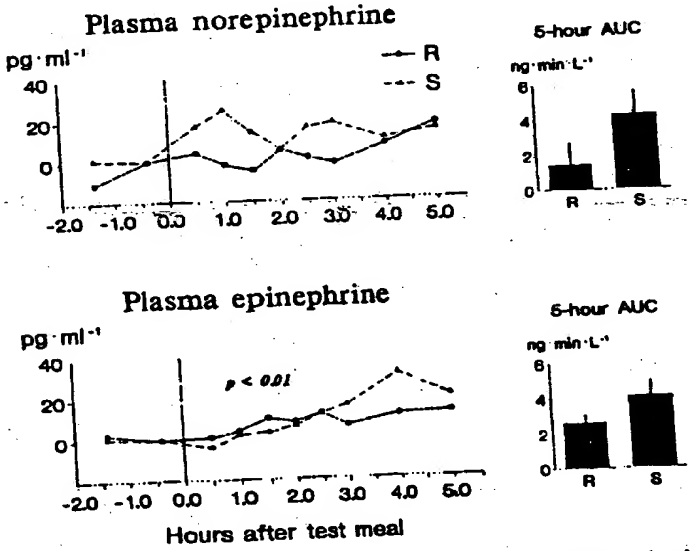

FIG 3. Changes in plasma concentrations of norepinephrine and epinephrine after a raw potato starch meal (54% resistant starch [RS]) (R) and a pregelatinized potato starch meal (0% RS) (S) in 10 healthy, normal-weight, male subjects. All data are mean (±SEM), expressed as differences from fasting concentrations. Plasma epinephrine (time effect: $F_{[9,144]} = 8.37$, $P < 0.0001$; interaction between diet and time: $F_{[9,144]} = 2.7$, $P = 0.0064$). Right panel: areas under the curves (AUC).

than would be expected from the difference in glucose load and glucose response after the two meals. In trying to explain this it may be useful to look at the changes in gastrointestinal hormones. Not only did the R meal result in a much lower blood glucose response compared with the S meal, but at the same time there seemed to be no effect on either the proximal (GIP) or distal (GLP-1) incretins in contrast with the increase in these hormones after the S meal. Both GIP and GLP-1 are known to be potent stimulators of insulin secretion (31). The large difference between the meals in these hormones may therefore explain the difference—beyond what can be expected from the glucose-stimulated insulin secretion—in insulin response between the two meals.

The subjective feelings of satiety and fullness were also significantly influenced by the digestibility of the starch. Thus, the pregelatinized and fully digestible potato starch meal resulted in greater feelings of satiety and fullness compared with the resistant and slowly digestible starch meal. Already, 1–1.5 h after the raw potato starch meal the subjective scores were back to fasting concentrations, whereas the satiating power of the S meal lasted 2.5–3 h postprandially.

It has previously been stated that changes in satiety after a carbohydrate load may be mediated through an effect of plasma glucose or hepatic glycogen concentration on specific glucosensitive cells in the brain (32) and studies have demonstrated a satiating effect of carbohydrate per se (14). We found no significant correlations between the changes in plasma glucose and in the four satiety scores. In a recent study from our department in which a high- and a low-fiber meal were given to 10 healthy subjects, the AUCs for glucose and satiety scores were not significantly correlated either (18). In the present study differences in hunger ratings between the two meals were in fact correlated with differences in Δpeak lactate concentration, which may reflect the rate of nonoxidative glucose disposal. In the present study carbohydrate was the only energy source in the test meals, but because an increased energy load in a meal has also been shown to result in an increased satiating power of the meal (33), it may be that the differences in satiety sensations were due to a different net energy load in the meals. A firmer conclusion may, however, require more direct measurements of glycogen stores or glucose disposal in order to explain changes in subjective satiety scores.

TABLE 4
Subjective evaluation of the test meal[1]

	R	S
Visual appeal (0: good, 10: bad)	5.3 ± 0.7	8.1 ± 0.5[2]
Taste (0: pleasant, 10: unpleasant)	6.0 ± 0.8	7.2 ± 0.8[3]
Aftertaste (0: none, 10: much)	5.4 ± 1.1	7.0 ± 0.7
Texture (0: firm, 10: loose)	7.7 ± 0.8	3.4 ± 0.8[4]
Palatability (0: good, 10: bad)	7.4 ± 0.6	8.2 ± 0.6

[1] $\bar{x}$ ± SEM. R, raw potato starch meal; S, pregelatinized potato starch meal.
[2–4] Significantly different from R (unpaired t test, $df = 9$): [2]$P = 0.007$, [3]$P = 0.02$, [4]$P = 0.0004$.

Gastrointestinal hormones have previously been connected with satiety and obesity (34, 35). In the present study some correlations were found between GIP and satiety scores but not between GLP-1 and satiety. The correlations found indicate that GIP may decrease satiety and increase hunger, which is in contrast with the previously suggested satiating power of GIP. In the present study, however, data on GIP were only available for five subjects. The correlations must therefore be considered with

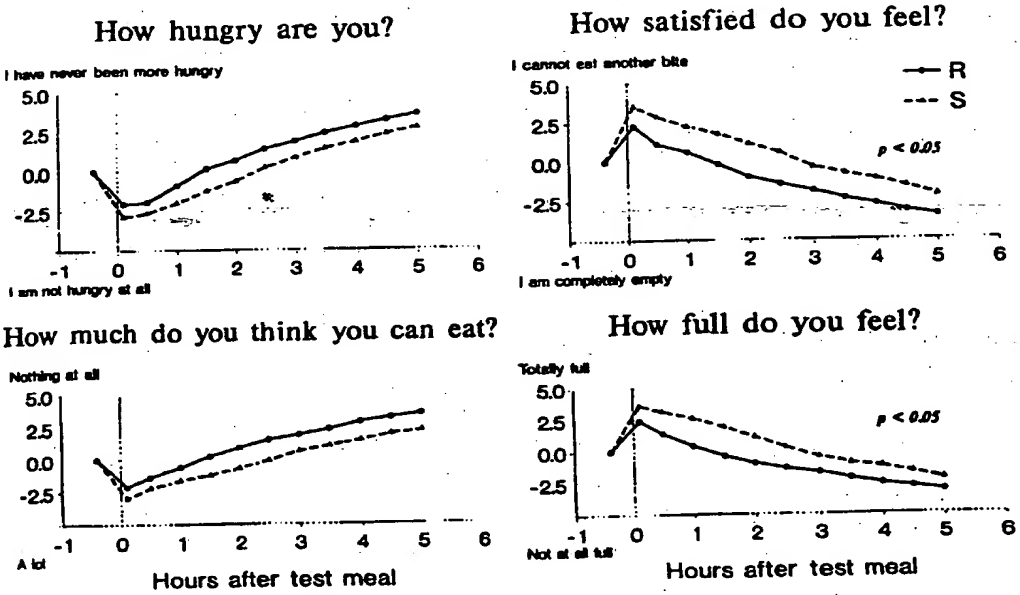

FIG 4. Subjective satiety scores after a raw potato starch meal (54% resistant starch [RS]) (R) and a pregelatinized potato starch meal (0% RS) (S) in 10 healthy, normal-weight, male subjects. All data are mean (±SEM), expressed as differences from fasting values. ANOVA: all (time effect: $F_{[11,198]}$, $P < 0.0001$); satiety (top right); diet effect: $F_{[1,18]} = 4.94$, $P = 0.04$; interaction between diet and time: $F_{[11,198]} = 1.95$, $P = 0.03$). Fullness (right bottom); interaction between diet and time: $F_{[11,198]} = 2.30$, $P = 0.012$).

some caution, and need to be confirmed before any further conclusions can be made.

Because the volume of the test meals and the time spent on consuming the meals on the 2 test days were similar, these factors cannot have influenced the satiating power of the two meals. However, the gelatinization of the S meal and the fact that it was a solid meal may have resulted in a reduction in gastric emptying rate (36) and in increased feelings of satiety and fullness after this meal compared with the liquid R meal. Also, the different ratings of taste, visual appeal, and texture may have influenced satiety and fullness ratings. In fact, positive correlations were found between aftertaste and satiety and fullness. This partly confirms the previous study by Hill et al (23) in which increased hunger was found with increased preference (palatability) of a meal. However, in their study there was no effect on fullness ratings of the meal preference (23).

The effect of RS on satiety shown in the present study may be considered a negative effect of RS with regard to appetite regulation and perhaps also weight control. Replacement of digestible starch with nondigestible starch in the diet may pose a risk of increasing the overall fat-to-carbohydrate ratio in the diet. Because a high-fat, low-carbohydrate diet has been shown to increase energy intake and body weight under ad libitum conditions, whereas a low-fat, high-carbohydrate diet has been shown to decrease spontaneous food intake and result in unexpected weight reductions (37–39), a replacement of digestible starch with RS may be undesirable in long-term weight regulation. Whether RS in a mixed meal has the same effect on appetite as in the present study remains, however, to be elucidated. Moreover, it is not possible to predict the effect on 24-h appetite sensations from the present 5-h results. The RS not digested in the small intestine will at a later stage, ie, 7–12 h after ingestion, be digested and fermented by bacteria in the large intestine, resulting in the production and uptake of short-chain fatty acids (5, 6, 40). Whether this may contribute to increasing satiety remains to be elucidated.

In conclusion, the intake of RS resulted in significantly lower postprandial plasma glucose, lactate, insulin, GIP, and GLP-1 responses and in a reduction of the satiating power compared with digestible starch. The changes in plasma glucose were not correlated with satiety or fullness, but correlations were found between GIP and satiety scores. Differences in texture and palatability of the test meals may also have influenced the subjective satiety ratings. Further studies are needed to clarify the effect of RS in a mixed meal, both acutely and long term.

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EFFECTIVE USE OF AUTOCLAVES
SafetyNet #26

Steam sterilization of materials is a dependable procedure for the destruction of all forms of microbial life. They are common laboratory tools that must be properly used to be effective. This establishes guidelines for the effective use of steam sterilizers (autoclaves) for the decontamination of cultures and other potentially biohazardous materials.

Successful Components of Sterilization
This should include validation of decontamination effectiveness. Validation of effectiveness includes monitoring temperature, pressure and cycle duration time for each cycle and providing periodic decontamination challenges (quality assurance), i.e. use of biological indicators. A logbook should be maintained to record autoclave use and be available for inspection.

- **Temperature-** This denotes heating in an autoclave employing saturated steam under a pressure of approximately 15 psi to achieve a chamber temperature of a least 121°C (250°F) for a minimum of 30 minutes.

- **Time-** The time is measured after the temperature of the material being sterilized reaches 121°C (250°F).

- **Contact-** Steam must contact all areas of the load. Autoclave bags should be left partially open during decontamination to allow steam to penetrate into the bag. Air pockets or inadequate steam supply will cause sterilization failure.

- **Containers-** Materials that are to be decontaminated should be carried to the autoclave in leak proof containers. Containers used to hold material while being autoclaved are described below.
  - Primary Containers – Clear autoclave bags come in a wide variety of sizes. They are usually placed in a secondary container during decontamination cycles to catch liquids that may drain out of the bag.
  - Secondary Containers – Plastic or stainless steel containers are commonly used to contain material during autoclaving. Polypropylene plastic pans with 6-12 inch sides are favored over polyethylene and polystyrene because it can withstand autoclaving without melting. Stainless steel containers are durable and a good conductor of heat.

- **Indicators-** These are tools used to validate the decontamination process.
  - Chemical indicators change color after being exposed to 121°C (250°F), but they have no time factor.
  - Tape indicators can only be used to verify that the autoclave has reached normal operating temperatures for decontamination.
  - Biological indicators are designed to demonstrate that an autoclave is capable of killing microorganisms. A load test using *Bacillus stearothermophilus* should be performed monthly.

- **Recordkeeping**- Records of maintenance, logs, calibration results and *Bacillus stearothermophilus* load tests should be kept for a minimum of three years.

Autoclave Training and Operation

Principal investigators, or supervisors must train and qualify their staff for operation of steam autoclaves for decontamination of materials. Qualified autoclave users should understand the time, temperature, pressure relationships required for proper materials decontamination. Additional training on handling materials to be decontaminated should also be provided. Supervisors should maintain a permanent record of training provided to their staff.

- Personnel should wear proper personal protective equipment, i.e. heat resistant gloves, eye protection and a lab coat, particularly when unloading the autoclave.

- Regularly inspect your autoclave components for proper operation. Follow the autoclave manufacturer's recommendations for inspections and service. Autoclaves should be inspected on a regular basis and temperature gauges should be calibrated at least annually. If a problem is found, promptly notify your area supervisor who will call for maintenance. **Do not operate an autoclave until it has been properly repaired.**

- **Never place sealed containers in an autoclave.** Large bottles with narrow necks can simulate sealed containers if filled with too much liquid.

- **Do not** autoclave items containing solvents, volatile or corrosive chemicals (phenol, trichloroacetic acid, ether, chloroform, etc.) or any radioactive materials. Call EH&S (752-1493) if you have questions regarding waste disposal.

- After loading and starting the autoclave, processing time starts after the autoclave reaches normal operating conditions of 121°C (250°F) and 15 psi pressure.

- Decontamination conditions vary with type of load therefore processing times will vary according to the conditions. A minimum of 30 minutes is needed to decontaminate biological waste.

- At the end of a decontamination cycle make sure that the pressure in the autoclave chamber is near zero before opening the door. Slowly crack open the autoclave door and allow the steam to gradually escape from within the autoclave.

- Take care when opening autoclave doors. Opening the autoclave door too quickly may result in glassware breakage and/or steam burns on your skin.

- Allow materials inside the autoclave to cool for 10 minutes before removing them from the autoclave.

- After autoclaving, waste can be disposed of as solid waste.

Wastes that are classified as medical waste (potentially/known infectious to humans and sharps waste) must be treated in accordance with the Medical Waste Management Act. For additional information, contact your EH&S Safety Advisor, EH&S at 752-1493 or ehs@ucdavis.edu.

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